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Facultad de Medicina
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Rutas de señalización celular activadas por receptores nicotínicos y su implicación en la neuroprotección y el dolor

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Trabajo presentado para optar al Título de Doctor
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Para que conste a efectos oportunos, expiden y firman la presente en Madrid a 14 de octubre de 2008.

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ÍNDICE

V. DISCUSIÓN GENERAL

VI. CONCLUSIONES

VII. BIBLIOGRAFÍA

I. JUSTIFICACIÓN

JUSTIFICACIÓN DE ESTA TESIS DOCTORAL

Esta tesis doctoral se enmarca en una de las líneas principales del Instituto Teófilo Hernando para la I + D del Medicamento (ITH), el estudio de los mecanismos de muerte neuronal y la búsqueda de fármacos neuroprotectores para prevenir o retrasar dicha muerte. En concreto, nuestro estudio se centra en los efectos neuroprotectores y las rutas de señalización que participan en la neuroprotección a través de la activación de los receptores nicotínicos en modelos de isquemia cerebral "in vitro". La enfermedad cerebrovascular es una alteración en el funcionamiento del cerebro debida a la disminución o interrupción del aporte sanguíneo al mismo por oclusión o hemorragia. Los accidentes cerebrovasculares son la causa del 9% del total de muertes en el mundo y son la segunda causa más común de muerte, después del infarto de miocardio (Donnan *et al.* 2008). Además, es la sexta causa más común de incapacidad en el mundo. El alto coste social y económico de la enfermedad cerebrovascular y sus secuelas impulsa la investigación dirigida a esclarecer los mecanismos fisiopatológicos responsables de la lesión del tejido cerebral sometido a isquemia.

Por otra parte, los agonistas de los receptores nicotínicos, sobre todo los derivados de epibatidina, han mostrado efectos antinociceptivos en distintos modelos de dolor. El efecto antinociceptivo de la epibatidina es 200 veces más potente que el de la morfina; dicho efecto no está mediado por receptores opioides. El inconveniente del uso de la epibatidina es que a las concentraciones a las que tiene este efecto antinociceptivo, están muy cercanas a las concentraciones tóxicas. El conocimiento de las rutas intracelulares que se activan a través del receptor nicotínico puede ayudar a comprender el/los mecanismos por los que los derivados de epibatidina tienen estos efectos antinociceptivos. Esta información puede ser de gran interés a la hora de diseñar y sintetizar nuevos fármacos que mejoren el perfil farmacodinámico y tóxico de la epibatidina. Esta es otra de las líneas de investigación principales del ITH.

Son estas ideas las que nos han llevado a realizar la investigación que se presenta en esta tesis doctoral, en la que evaluamos el efecto neuroprotector y analgésico de distintos agonistas del receptor nicotínico.

II. INTRODUCCIÓN

1. RECEPTORES NICOTÍNICOS DE ACETILCOLINA

1.1. Estructura, composición y función de los nAChR

Los receptores nicotínicos de acetilcolina pertenecen a la superfamilia de canales iónicos regulados por ligando y se localizan tanto en el sistema nervioso central como en el sistema periférico. Los nAChRs son glucoproteínas transmembrana de 270-290 KDa que se componen de 5 subunidades insertadas en la membrana lipídica; cada una de estas subunidades consta de 4 hélices α transmembrana denominadas M1, M2, M3 y M4 (Corbin *et al.* 1998). Hasta ahora se han identificado 9 subunidades α (α2-α10) y 3 subunidades β (β2-β4), codificadas por genes distintos. Estas subunidades son capaces de unirse entre sí con diferentes estequiometrias dando lugar a receptores funcionales (Figura 1). La subunidades α7, α8, α9 y α10 son capaces de formar receptores funcionales sin necesidad de otra subunidad distinta (receptores homoméricos, p.ej.: α7); sin embargo, las subunidades α2-α5 necesitan de las subunidades β2-β4 para formar receptores funcionales (receptores heteroméricos, p.ej.: α3β4, α4β2) con una estequiometría α(2)β(3) (Cooper *et al.* 1991).

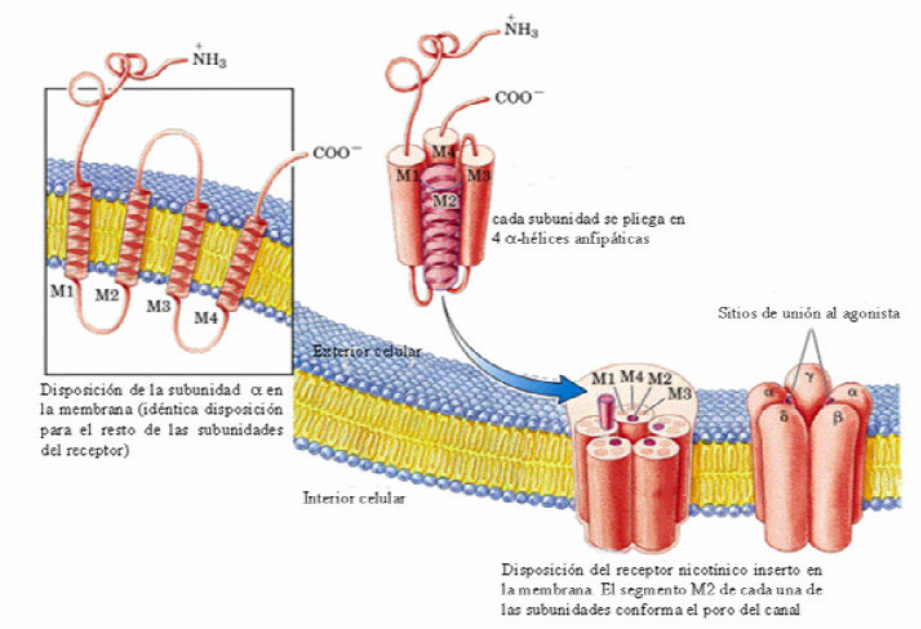

Figura 1. Estructura del receptor nicotínico de acetilcolina.

Las neuronas expresan gran diversidad de nAChR que, junto a los receptores de tipo muscarínico, son capaces de elevar la concentración de calcio citosólico ($[Ca^{2+}]_c$) por su activación con el agonista endógeno, la ACh. La localización subcelular de estos receptores, así como su sensibilidad al ligando y sus características cinéticas, condicionan el incremento de la $[Ca^{2+}]_c$ y su posible función dentro de la célula. En los últimos años, se han identificado claramente algunas de las funciones fisiológicas y/o fisiopatológicas de los nAChR, que se describen a continuación.

En el individuo adulto se ha descrito la implicación de los nAChR en multitud de funciones del sistema nervioso: el aprendizaje y la memoria, la atención, el control de la actividad motora, la percepción sensorial y del dolor o la regulación corporal de la temperatura. Generalmente estos efectos son debidos a la existencia de receptores nicotínicos que contienen la subunidad α7 en la terminación presináptica, que actúan modulando la secreción de neurotransmisores (Wonnacott 1997). Sin embargo, no hay que olvidar el papel de estos receptores a un nivel postsináptico, en el control de la transmisión en ganglios periféricos, hipocampo y corteza sensorial (Jones *et al.* 1999).

También se ha descrito la existencia de transcripción de diversas subunidades del receptor nicotínico a lo largo del desarrollo embrionario, lo que le confiere un papel más o menos influyente en la proliferación y diferenciación celular.

Los nAChR también están implicados en procesos de dependencia a la nicotina o a otras drogas. En este caso, son los receptores $\alpha4\beta2$ presinápticos los que modulan la neurotransmisión de dopamina en el sistema mesolímbico (Picciotto *et al.* 1998, Marubio *et al.* 2003, Tapper *et al.* 2004).

En los últimos años, se ha postulado la implicación de estos receptores en una gran variedad de patologías neuronales: enfermedades neurodegenerativas como el Alzheimer, Parkinson o demencia por cuerpos de Lewy (Zanardi *et al.* 2002), disfunciones cerebrales como el autismo o la esquizofrenia (Freedman *et al.* 2000) o la epilepsia (Combi *et al.* 2004). En el caso de las enfermedades neurodegenerativas, la implicación de los nAChRs como mediadores de procesos de neuroprotección está cobrando gran interés como estrategia terapéutica. En concreto, la neuroprotección se ha relacionado con los nAChR del subtipo α7, ya que se sabe que los agonistas de estos receptores reducen la citotoxicidad mediada por glutamato o inducen la síntesis de factores de crecimiento nervioso (Shimohama *et al.* 1996, Kaneko *et al.* 1997).

Estos receptores, además de encontrarse en neuronas, se han encontrado en muchas células no excitables, como

queratinocitos de la piel (Grando 1997), células del endotelio vascular (Conti-Fine *et al.* 2000), macrófagos (Wang *et al.* 2003), y linfocitos T y B (Kawashima & Fujii 2000, Skok *et al.* 2003, Skok *et al.* 2005). El hecho de que estas células expresen nAChR, sugiere que deben tener alguna función. En este sentido, tanto la subunidad β2 como la α7 participan en el desarrollo y la activación de linfocitos B en médula ósea y en bazo, respectivamente (Skok *et al.* 2007). Como dato más interesante, cabe destacar el papel del subtipo α7 en los procesos inflamatorios (Wang et al. 2003).

1.2. Los receptores nicotínicos: dianas terapéuticas para el desarrollo de nuevos fármacos

Nos hemos centrado en dos enfermedades concretas, la isquemia cerebral y el dolor, patologías en la que la activación de los nAChR pueden mediar neuroprotección y analgesia. A continuación se describirán en mayor detalle cada una de las citadas patologías y cómo la actuación sobre receptores nicotínicos puede contribuir a la mejora de las mismas:

2. LA ISQUEMIA CEREBRAL

2.1. Fisiopatología de la isquemia cerebral

Los accidentes cerebrovasculares son la causa del 9% del total de muertes en el mundo y son la segunda causa más común de muerte, después del infarto de miocardio (Donnan *et al.* 2008). En 2002, la incapacidad debida a los accidentes cerebrovasculares se consideró la sexta causa más común de incapacidad en el mundo. Teniendo en cuenta el aumento en la calidad y en la esperanza de vida media de la población, se estima que en el año 2030 será la cuarta causa principal de incapacidad (Donnan et al. 2008). Así, los accidentes cerebrovasculares son actualmente la entidad patológica que presenta más desafíos científicos y terapéuticos (Durukan & Tatlisumak 2007).

Los accidentes cerebrovasculares pueden ser isquémicos o hemorrágicos. La isquemia cerebral constituye el 80% de los accidentes cerebrovasculares (Thrift *et al.* 2001); normalmente se debe a una oclusión embólica o trombótica de una arteria cerebral que, con mucha frecuencia, es la arteria cerebral media o sus ramificaciones (Durukan & Tatlisumak 2007). De esta manera, se produce una reducción del flujo sanguíneo cerebral, cuya consecuencia primaria es la falta de oxígeno y glucosa necesarios para el metabolismo cerebral.

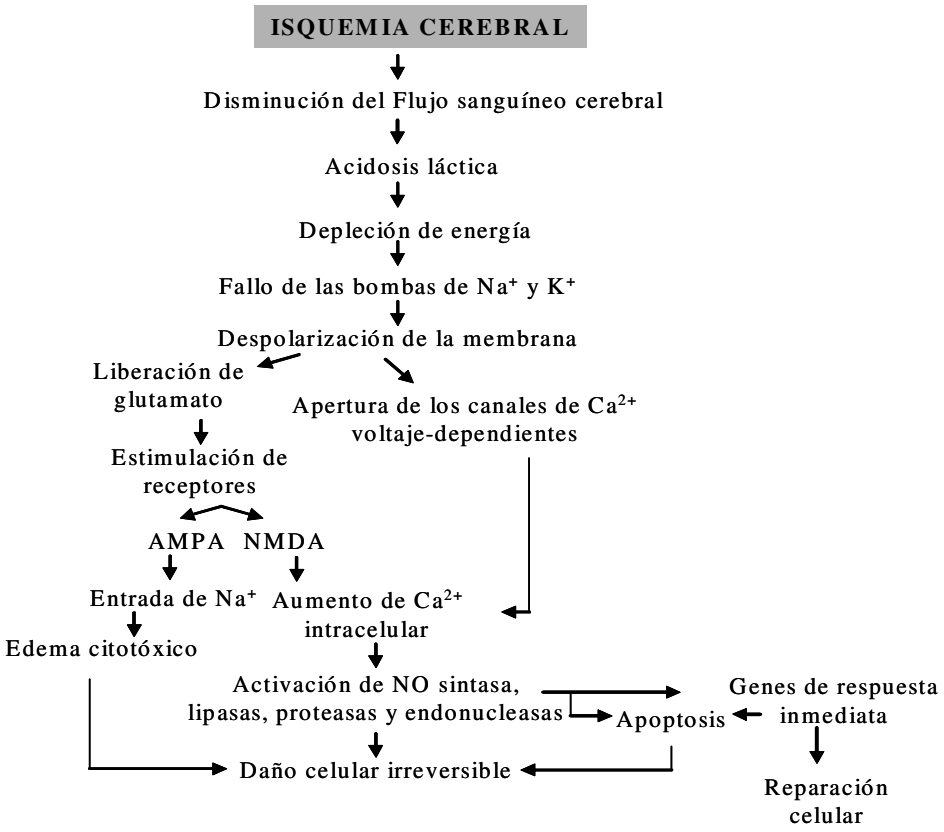

Figura 2. Esquema que representa la cascada isquémica: eventos bioquímicos que conducen a la destrucción del parénquima cerebral en neuronas, glía y en el componente vascular.

En el cerebro existe un flujo sanguíneo cerebral casi constante de 55 cc/100 g/min. Tras la oclusión de un vaso, el flujo sanguíneo cerebral disminuye por debajo de 10-15 cc/100 g/min y se produce una muerte celular rápida característica del denominado núcleo del infarto. Las neuronas en este área mueren por necrosis. En los alrededores del núcleo del infarto, existe un área con perfusión cerebral críticamente reducida (< 22 cc/100 g/min), que se denomina zona de penumbra isquémica. En este área, el consumo de oxígeno todavía es suficiente, aunque hay una disminución del metabolismo, y las neuronas no son capaces de disparar potenciales de acción, pero tienen energía suficiente como para mantener el potencial de membrana, por lo que son neuronas no funcionales, pero

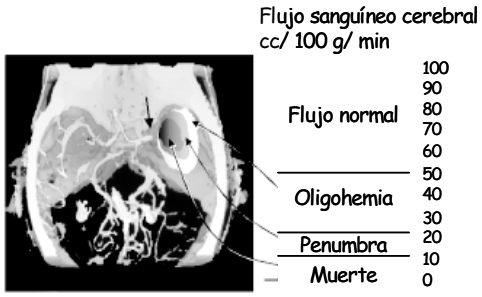

Figura 3. Diagrama que representa el gradiente de presiones generado tras la obstrucción de la arteria cerebral media (flecha corta).

potencialmente viables, y por tanto recuperables (Figura 3).

En el cerebro solo existen reservas energéticas para cubrir los requerimientos energéticos cerebrales durante 1 minuto; así, la primera consecuencia de la reducción del flujo sanguíneo cerebral es la acumulación de lactato y la disminución del pH (acidosis láctica) a través de la glucolisis anaerobia. La acidosis láctica puede empeorar la producción de especies reactivas de oxígeno (ROS, del inglés "reactive oxygen species"), interferir con la síntesis proteica y empeorar el daño isquémico (Mergenthaler *et al.* 2004). El resultado es una disminución en los niveles de ATP, ya que el metabolismo oxidativo está comprometido. En el núcleo del infarto los niveles de ATP disminuyen entre un 80-85% (Folbergrova *et al.* 1995).

Para su buen funcionamiento, la célula ha de mantener sus niveles de $[Ca^{2+}]_c$ libre en torno a 100 nM. Para ello, las neuronas poseen dos mecanismos principales para extraer el Ca^{2+} del interior celular: la Ca^{2+}-ATPasa y el intercambiador Na^+/Ca^{2+} (NCX, del inglés Na^+/Ca^{2+} exchanger). El NCX no necesita energía para realizar su función, pero depende de la Na^+/K^+-ATPasa para mantener el gradiente de Na^+. Así, la depleción de ATP hace que estas bombas dependientes de ATP no funcionen bien; se produce así un aumento de Na^+, Ca^{2+} y Cl^- intracelular y de K^+ extracelular. Como consecuencia de esta desregulación en la homeostasia iónica, se produce una rápida despolarización hasta 0 mV, también denominada despolarización anóxica, que es característica en las neuronas que sufren un insulto isquémico (Pringle 2004, Durukan & Tatlisumak 2007). Esta alteración en la homestasia iónica produce tanto edema citotóxico, característico de la muerte celular por necrosis, como la apertura de canales de calcio dependientes de voltaje, la entrada de Ca^{2+} y la liberación por exocitosis de glutamato.

El glutamato es el principal neurotransmisor excitador. En condiciones fisiológicas normales, a concentraciones milimolares no es tóxico (Liu *et al.* 1996). Sin embargo, en un cerebro isquémico, se dan ciertas condiciones que aumentan la toxicidad del glutamato. La despolarización anóxica tiene dos efectos; primero, produce un aumento de la $[Ca^{2+}]_c$ en la neurona presináptica que induce la liberación de glutamato, y este glutamato puede activar receptores NMDA de la neurona postsináptica ya que esta despolarización anóxica produce la liberación del Mg^{2+} que se encuentra bloqueando el receptor NMDA en condiciones normales; y segundo, el aumento de Na^+ intracelular y de la acidosis extracelular produce un bloqueo del transportador de glutamato en astrocitos (Szatkowski & Attwell 1994, Swanson *et al.* 1995). Esto hace que aumente la concentración de glutamato libre y que haya

una activación masiva de receptores NMDA y no-NMDA.

La activación de los receptores de glutamato del tipo AMPA/Kainato también se han implicado en la fisiopatología de la isquemia cerebral. De hecho, distintos antagonistas de los receptores AMPA como el NBQX reducen el volumen de infarto cerebral en modelos de isquemia cerebral focal (Gill 1994). Además, los receptores AMPA son más permeables a Na^+ que a Ca^{2+} debido a la presencia, en condiciones normales, de la subunidad GluR2, que limita la entrada del Ca^{2+} a través de los receptores AMPA (Mishina *et al.* 1991). De hecho, esta subunidad del receptor AMPA disminuye tras la isquemia cerebral total en rata, haciendo que el receptor AMPA deje pasar más Ca^{2+}.

Todos estos mecanismos contribuyen a una elevación de la $[Ca^{2+}]_c$ que provoca la activación de cascadas metabólicas como, por ejemplo, la activación de proteasas y endonucleasas, la producción excesiva de ROS y, en definitiva, la ruptura de la función mitocondrial y la muerte celular.

La producción de ROS es, en gran parte, la responsable del daño producido durante la reperfusión isquémica. En circunstancias normales, un 5% del oxígeno consumido por la cadena respiratoria mitocondrial es transformado en ROS (principalmente en forma de radical superóxido, O_2^-); éstos son neutralizados por los mecanismos de defensa antioxidantes intracelulares como la superóxido dismutasa (SOD) y la catalasa. La SOD cataliza la reacción de transformación del anión superóxido en peróxido de hidrógeno (H_2O_2) y la catalasa transforma el H_2O_2 en agua y oxígeno. La lesión celular se produce cuando la producción de ROS sobrepasa la capacidad de defensa de los antioxidantes intracelulares. Esta situación se produce durante la reperfusión isquémica.

Por otra parte, durante la isquemia se produce la depleción de los niveles de ATP, aumentando los de ADP y AMP y la formación de hipoxantina (Figura 4). Al mismo tiempo, la isquemia produce la transformación de xantina dehidrogenasa en xantina oxidasa debido a la activación de Ca^{2+}-calmodulina (CaM) por la sobrecarga de calcio que se produce en este periodo.

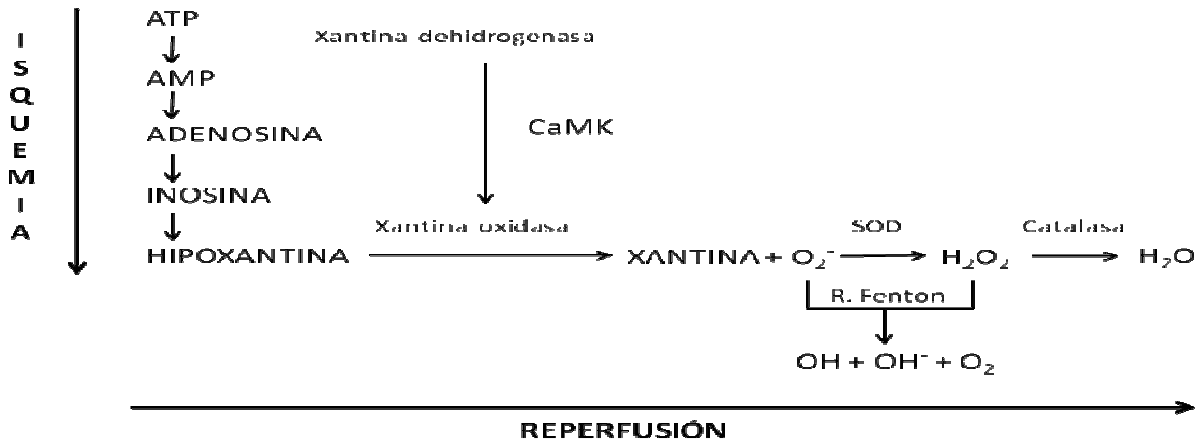

Figura 4. Esquema bioquímico de la isquemia-reperfusión

De esta forma, en cuanto se produce la reperfusión, la presencia de oxígeno activa la reacción que produce la xantina oxidasa, convirtiendo la hipoxantina acumulada durante la isquemia en xantina, a través de la vía del ácido úrico, produciendo grandes cantidades de O_2^-. La producción masiva de este anión, además de inactivar distintas enzimas, es precursor del H_2O_2, que es un oxidante potente relativamente estable. La acumulación de H_2O_2 en presencia de hierro u otros metales como manganeso o cobre, desencadena la reacción de Haber-Weiss catalizada por hierro, descrita por Fenton (Figura 5), en la que el producto de la reacción es el radical hidroxilo (OH^-) que es muy ine stable y altamente reactivo.

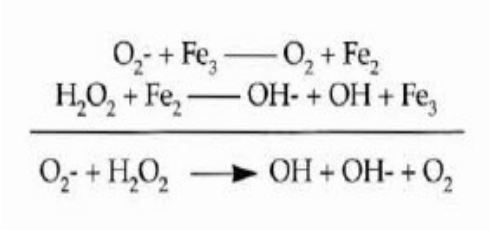

Figura 5. Esquema de la reacción de Fenton

El óxido nítrico (NO) es un mensajero químico cuya síntesis cataliza la óxido nítrico sintasa (NOS, del inglés Nitric oxide synthetase) a partir del aminoácido L-arginina. Se conocen tres isoformas de la enzima NOS: la endotelial (eNOS) y la neuronal (nNOS), que son constitutivas y se regulan por Ca^{2+}-calmodulina, y la inducible (iNOS), cuya transcripción se activa con la respuesta inflamatoria. El NO ejerce acción vasodilatadora, regula la neurotransmisión y la plasticidad sináptica e interviene en procesos de conducta. Durante una isquemia cerebral, se origina una elevada producción de NO, cuyo papel es controvertido ya que se relaciona tanto con neuroprotección como con neurodegeneración. El NO se une a la citocromo c oxidasa, que constituye el complejo IV de la cadena respiratoria mitocondrial, compitiendo con el oxígeno e inhibiendo la respiración. El NO, además, puede dar lugar a derivados reactivos como

NO_2, NO_3 o S-nitrosotioles que pueden reaccionar con el $O_2^{\cdot-}$ dando lugar al peroxinitrito ($ONOO^-$), un potente oxidante.

Así, la consecuencia final de la génesis excesiva de ROS es el daño en el ADN, la peroxidación lipídica de la membrana plasmática, la desnaturalización de proteínas y, en definitiva, una disfunción celular que lleva a la muerte celular.

2.2. Tipos de muerte celular

Como consecuencia de la isquemia y de la reperfusión, se sucede una serie de acontecimientos bioquímicos que desembocan en la muerte neuronal, que puede ocurrir de forma rápida e incontrolada (necrosis), o bien puede ser programada por la propia célula, desencadenando un proceso activo que conduce al suicidio celular (apoptosis) o muerte celular programada (Figura 6). La muerte por necrosis se acompaña de hinchazón de las organelas celulares y edema celular, con la consiguiente ruptura de las membranas celulares y lisis, con vertido de los componentes intracelulares al espacio extracelular, causando edema en el tejido e induciendo una respuesta inflamatoria. La muerte celular programada, en cambio, es un proceso controlado por la célula que puede desarrollarse siguiendo distintas vías que dan lugar a varios tipos, en términos morfológicos y bioquímicos, de muerte celular programada.

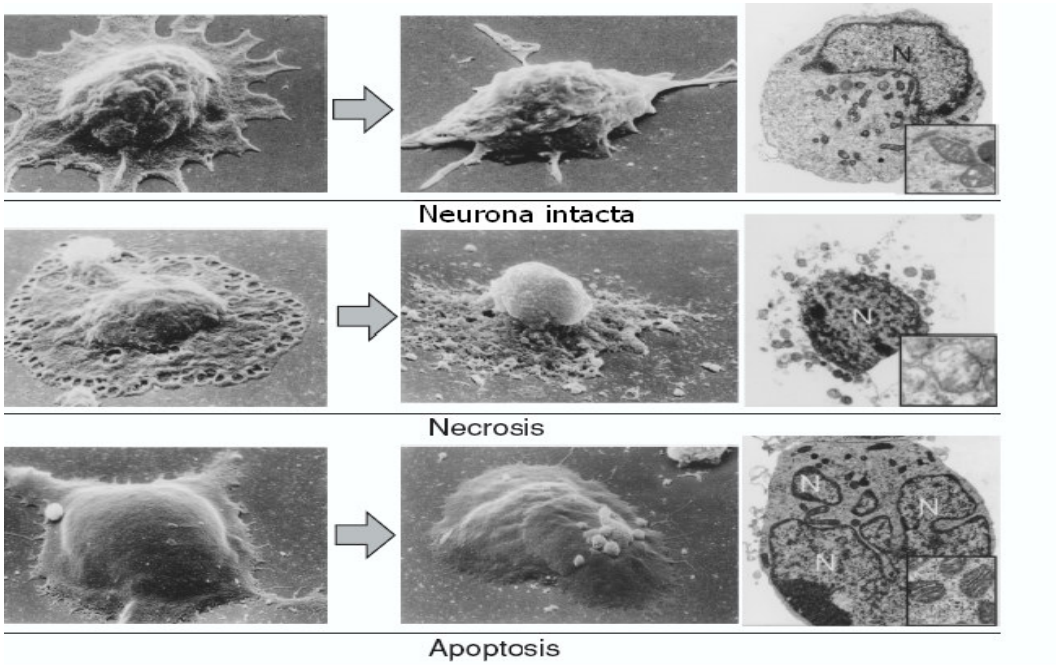

Figura 6. Diferencias morfológicas entre necrosis y apoptosis a través de microscopia electrónica por barrido observada a las 6 h (panel de la izquierda) o 12 h (panel central) tras el comienzo del cultivo y microscopia electrónica por transmisión (panel derecha). El panel de la derecha muestra un aumento de la mitocondria. La células normales son neuronas en cultivo con suero, las necróticas están cultivadas sin suero y las apoptóticas sin suero más una alta concentración de glucosa. Adaptado de (Ueda *et al.*, 2004).

La apoptosis puede ser desencadenada desde la mitocondria, la activación de los llamados receptores de muerte o desde el retículo endoplásmico por el llamado estrés reticular (ER) (Ueda & Fujita 2004). La apoptosis es el tipo de muerte celular programada mejor caracterizado. Morfológicamente las células se redondean y forman burbujas, la cromatina se condensa y se fragmenta y se forman los cuerpos apoptóticos. El fosfolípido de membrana fosfatidilserina, que se encuentra normalmente en la monocapa interna de la membrana citoplasmática, cuando la célula entra en apoptosis también se encuentra en la monocapa externa (Fadok *et al.* 1992). Estos cambios morfológicos y bioquímicos son el resultado de la activación de una serie de cisteín-aspartato-proteasas, las caspasas (Fadok et al. 1992, Thornberry & Lazebnik 1998).

La mitocondria posee mecanismos de transporte específicos para el Ca^{2+}, pudiendo entrar por el uniportador; el Ca^{2+} se bombea hacia fuera por el intercambiador Na^{+}/Ca^{2+} mitocondrial (Gunter *et al.* 2000). El intercambiador se satura cuando los niveles de calcio en la mitocondria aumentan demasiado, pero el uniportador no es saturable con el incremento extracelular del catión. La consecuencia es que cuando la concentración extramitocondrial de Ca^{2+} aumenta demasiado, la mitocondria ya no puede regular la concentración de calcio interna, y por lo tanto, se sobrecarga de este catión (Gunter et al. 2000).

En condiciones fisiológicas, esta sobrecarga puede suceder sin ningún daño mitocondrial. De hecho, en la célula cromafín estimulada con ACh, K^{+} o cafeína la mitocondria puede acumular grandes concentraciones de Ca^{2+} (Montero *et al.* 2000) que sirven para regular las señales de $[Ca^{2+}]_c$ y la exocitosis (Cuchillo-Ibanez *et al.* 2004). Sin embargo, cuando la sobrecarga va acompañada de la combinación de otros factores celulares (el estrés oxidativo, concentraciones altas de fosfatos o bajas concentraciones de adenina) la mitocondria entra en un estado llamado de permeabilidad transitoria. El estado de permeabilidad transitoria de la mitocondria consiste en la apertura de un poro en la membrana interna, conocido como MPTP (del inglés, *mitocondria permeability transition pore*), ocasionando una permeabilidad no especifica de la membrana para cualquier molécula menor que 1.5 kDa. Si esta situación no es revertida, lleva a la muerte neuronal por necrosis debido a un fracaso energético (Halestrap 2006).

La consecuencia de esta apertura del poro de transición es la liberación del citocromo c, y otras moléculas relacionadas con la apoptosis como el AIF, Smac/DIABLO, EndoG y HtrA2/Omi, la consecuente

activación de la caspasa 3 y la ejecución de la apoptosis a través de la fragmentación del ADN (Halestrap *et al.* 2004, Ueda & Fujita 2004).

Las conocidas proteínas de la familia Bcl-2 pueden regular la apoptosis positiva o negativamente mediante una regulación de la apertura del MPTP (Figura 7). Entre ellas se encuentran Bax y Bad que la estimulan y Bcl-2 y BcL-xL que la inhiben cuando se sobrexpresan (Ueda & Fujita 2004). Muchas otras proteínas de esta familia regulan la apertura del poro, por ejemplo Bad y Bim, que pueden estimular la apertura del poro cuando se unen a Bcl-2 o Bcl-xL (Tsujimoto 2002).

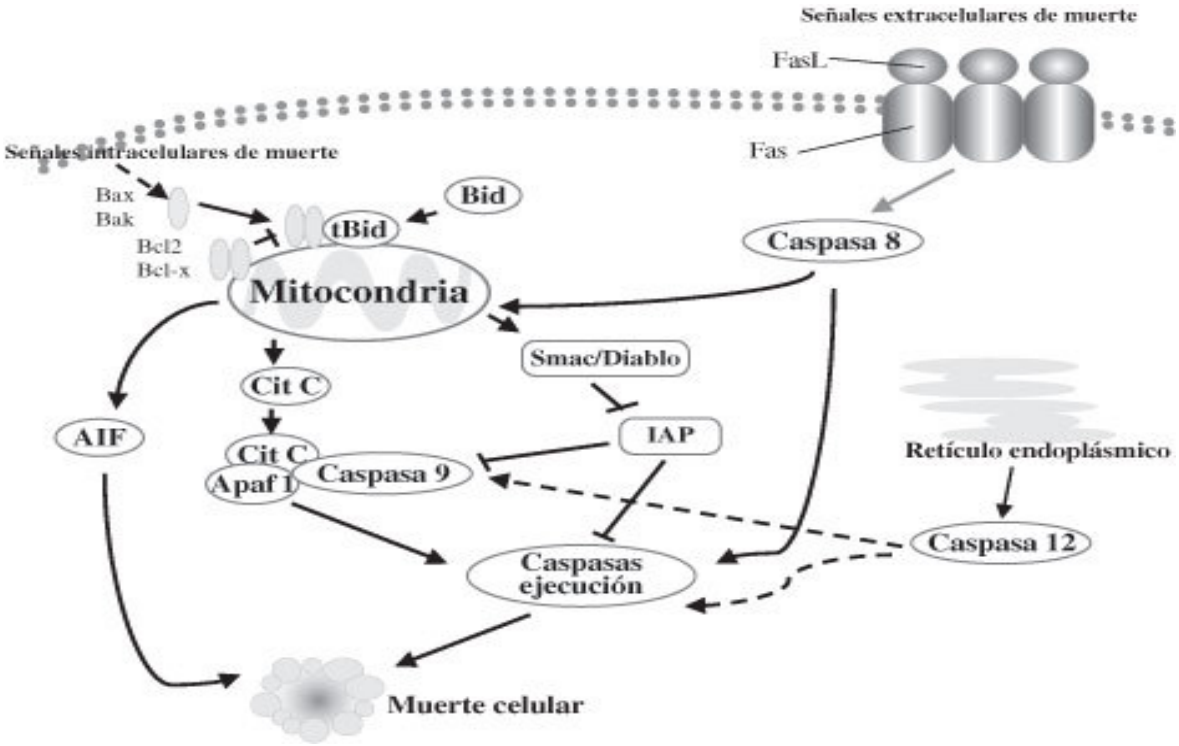

Figura 7. Esquema que representa las vías de muerte celular apoptótica.

Otro mecanismo que desencadena la apoptosis es la activación de los receptores de muerte Fas y TNFα-1, que están implicados en las señales de muerte inducida por ligandos como el Fas-L o TNFα, mediante los dominios proteicos de muerte FADD, RIP y TRADD, seguido de la activación de la caspasa-8 y caspasa-3 (Ueda & Fujita 2004).

El tercer mecanismo que puede desencadenar la apoptosis proviene del retículo endoplásmico. El estrés reticular, inducido por la salida de calcio de su interior,

induce el acúmulo de proteínas aberrantes en su interior (Ueda & Fujita 2004).

2.3. Receptores nicotínicos y neuroprotección

Hay múltiples estudios realizados en cultivos primarios y en distintos modelos *in vivo*, que demuestran que la nicotina y otros agonistas o moduladores de los nAChR son protectores en diversos modelos que remedan diversas enfermedades.

Así, la nicotina es neuroprotectora frente a la toxicidad inducida por glutamato en diversos modelos celulares tales como las neuronas corticales (Akaike *et al.* 1994) y en células de feocromocitoma de rata (PC12) (Sun *et al.* 2004). Además de proteger frente a glutamato, la nicotina es capaz de ofrecer neuroprotección frente a distintos estímulos tóxicos, como la privación de factores tróficos en PC12 (Yamashita & Nakamura 1996), β-amiloide en neuronas (Kihara *et al.* 1997), o la hipoxia en cultivos de neuronas corticales (Hejmadi *et al.* 2003). No solo los agonistas nicotínicos son neuroprotectores en modelos *in vitro* sino que, además, los moduladores alostéricos de los nAChR también lo son. La galantamina, un inhibidor competitivo de la acetilcolinesterasa y potenciador alostérico del nAChR, es neuroprotectora en distintos modelos: frente a la lesión inducida por ácido okadaico y β-amiloide en células de neuroblastoma humano SHSY-5Y (Arias *et al.* 2004, Arias *et al.* 2005); frente a la muerte neuronal inducida por glutamato (Takada-Takatori *et al.* 2006); o frente a la lesión inducida por glutamato más β-amiloide en cultivos primarios de neuronas corticales (Kihara *et al.* 2004).

Los agonistas de los nAChR también son neuroprotectores en diferentes modelos de lesión cerebral *in vivo*. Por ejemplo, el GTS-21 (un agonista selectivo α7) y la nicotina, disminuyeron los déficits cognitivos tras una isquemia cerebral en ratas, aunque sólo el GTS-21 redujo de manera significativa la muerte neuronal (Nanri *et al.* 1997, Nanri *et al.* 1998). Sin embargo, en otro modelo de isquemia cerebral en ratas, la nicotina sí protegió frente a la lesión neuronal en la capa CA1 del hipocampo (Kagitani *et al.* 2000). Además de en estos modelos de isquemia, los agonistas nicotínicos han mostrado neuroprotección frente a la lesión inducida por metanfetamina (Maggio *et al.* 1998) o frente a la lesión provocada mediante tratamiento crónico con MPTP (Zoli *et al.* 1999). La galantamina también tiene acciones neuroprotectoras en modelos in vivo. Así, la galantamina inyectada 3 horas después de inducir una isquemia cerebral global en jerbos, protegió las neuronas de la capa CA1 del hipocampo. Además, esta neuroprotección se correlacionó con la recuperación de la pérdida de memoria de

los animales sometidos a isquemia (Lorrio *et al.* 2007).

3. EL DOLOR

3.1. Neurotransmisión del dolor

El dolor está incluido entre los mecanismos de control homeostático que posee el organismo, ya que actúa como un mecanismo de alerta para informar que algo amenaza la integridad física y mantiene nuestra atención en torno a la identificación de su causa y la eliminación de la misma. El dolor agudo tiene la función de proteger inmediatamente del estimulo nocivo; el dolor subagudo produce una inactividad temporal que tiene por objeto recuperar al organismo o tejido del daño ocasionado. El dolor persistente que acompaña una lesión inflamatoria o neural, se caracteriza por su naturaleza espontánea (no depende del estímulo), va acompañado de hiperalgesia (aumento del dolor producido por un estimulo algógeno) y alodinia (la sensación de dolor ante un estimulo de baja intensidad, que normalmente no la produce). El dolor crónico, que dura meses o años, se considera un proceso no adaptativo y sin propósito fisiológico, y por lo tanto, patológico (Millan 1999).

La percepción del dolor es compleja e implica no solo la transducción de un estímulo nocivo, sino también procesos emocionales y cognitivos que lo acompañan (Julius & Basbaum 2001). Así, se puede decir que el dolor está influenciado tanto por factores fisiológicos como psicológicos, y por ello, en animales solo puede ser evaluado de manera indirecta. El componente fisiológico y medible del dolor en animales se denomina nocicepción, y por ello, los modelos animales de algesia son modelos de nocicepción.

En cuanto al origen del dolor, existen cuatro tipos principales de dolor: i) El “dolor nociceptivo”, que se origina tras la estimulación excesiva de los nociceptores localizados en la piel, vísceras u otros órganos. ii) El “dolor neurogénico”, que refleja el daño de tejido neuronal en la periferia o en el sistema nervioso central (“dolor central”). iii) El “dolor neuropático”, que sucede tras una disfunción o daño de un nervio o grupo de nervios. iv) El “dolor psicógeno”, que no procede de una fuente somática identificable y que puede estar reflejando factores psicológicos (Millan 1999).

La secuencia de los eventos que conducen a la integración de un estímulo doloroso implica receptores periféricos (nociceptores), que están ampliamente distribuidos en la piel, vasos, músculos, articulaciones y vísceras. Los nociceptores son receptores sensibles a diferentes estímulos, que pueden ser térmicos, mecánicos o químicos. De todos ellos, la señalización química es la más común y la que representa las más diversas formas de

generación de señal en las neuronas sensitivas (Besson 1999, Besson & Chaouch 1987, Millan 1999). Los nociceptores son las terminaciones nerviosas de las neuronas aferentes primarias, que se encuentran en los ganglios de la raíz dorsal de la médula espinal. El axón de estas neuronas se bifurca para enviar prolongaciones a la médula espinal e inervar los tejidos. Los aferentes primarios se clasifican de acuerdo con criterios funcionales y anatómicos, entre ellos la velocidad de conducción, el diámetro y el grado de mielinización (Cross 1994, Julius & Basbaum 2001, Millan 1999, Pleuvry & Lauretti 1996); según estos criterios se habla de fibras Aα, Aβ, Aδ y C.

El área primaria receptora de la mayoría de las informaciones somatosensoriales es el asta dorsal de la médula espinal (Coggeshall & Carlton 1997), una estructura dividida en láminas. Las neuronas nociceptivas específicas están situadas en las láminas más superficiales del asta dorsal (lámina I o zona marginal y lámina II o sustancia gelatinosa), aunque también se encuentran en láminas más profundas (láminas V, VI, VII y X). Estas neuronas responden a estímulos periféricos de gran intensidad y sus fibras aferentes primarias son principalmente de los tipos Aδ y C (Calvino & Grilo 2006). Los axones de las neuronas nociceptivas del asta dorsal constituyen el tracto espinal ascendente que proyecta su información a distintos niveles supraespinales. La mayoría de estos axones se cruzan en el segmento espinal, siguiendo su camino por el lado contralateral de la médula espinal. De esta forma, las proyecciones supraespinales son contralaterales al estímulo. Las neuronas sensoriales secundarias reciben sus señales sensoriales mediante la liberación de glutamato y SP de los aferentes primarios (Hill 2001). Las vías ascendentes más importantes son: el tracto espinotalámico, el tracto espinoreticular y el tracto espinomesencefálico, entre otros. Finalmente, la integración del estímulo nociceptivo se realiza en neuronas localizadas principalmente en dos zonas del tálamo: 1) las neuronas del tálamo ventroposterolateral, que proyectan sus axones hacia áreas somatosensoriales S1 y S2 de la corteza parietal, donde se descifran las características de la señal nociceptiva y se genera la percepción del dolor (calidad, localización, intensidad y duración). 2) Las neuronas del tálamo medio proyectan sus axones hacia la corteza frontal, corteza insular y corteza cingular anterior, que generan las respuestas emocionales más complejas del dolor (Calvino & Grilo 2006).

La información nociceptiva se integra con experiencias del pasado y se procesa para producir la percepción del dolor y promover la respuesta adecuada que es enviada hacia la médula espinal a través de las neuronas descendentes (Besson 1999,

Millan 1999). La modulación descendente de la información nociceptiva implica distintos sistemas de neurotransmisores entre los cuales podemos mencionar los sistemas opioide, serotonérgico, noradrenérgico, gabaérgico, glutamatérgico, además de de los cannabinoides endógenos, entre otras. Estas vías tienen sus terminaciones en la sinapsis entre la neurona primaria y secundaria en la médula y tienen un efecto final inhibidor sobre la información ascendente (Millan 2002).

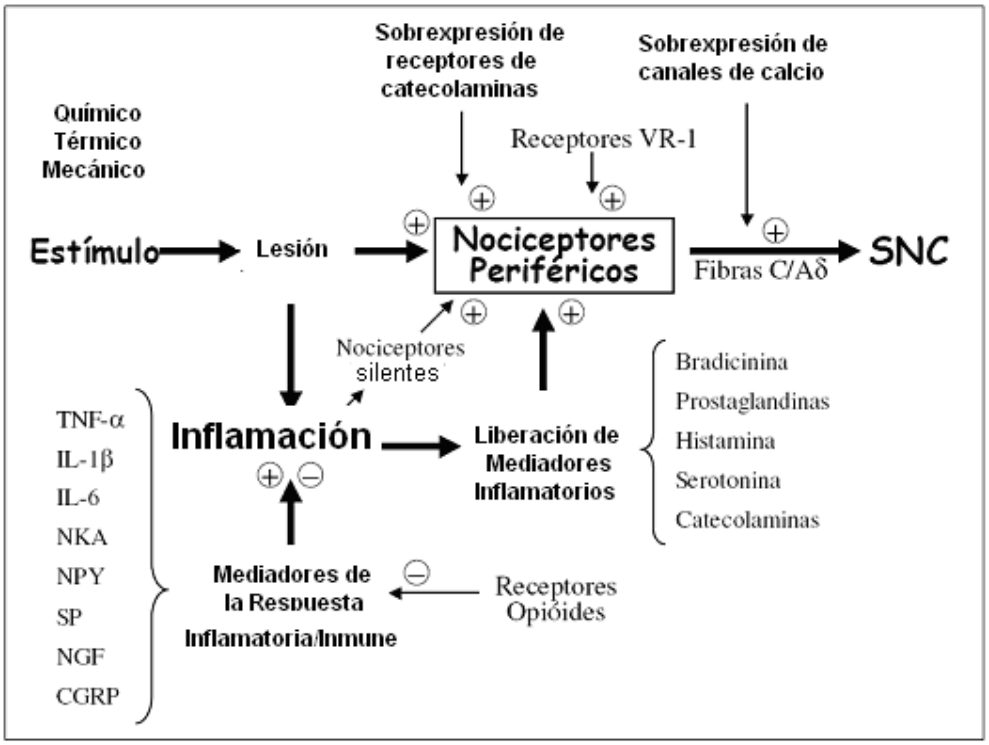

Figura 8. Factores responsables de la activación de los nociceptores periféricos. Adaptado de Hill (2001).

La actividad y el metabolismo de estas fibras también se regulan por mecanismos endógenos, como los productos de la lesión tisular y los mediadores inflamatorios. Tras una lesión tisular, estos mediadores pueden ser liberados por las neuronas sensoriales y simpáticas y, por las células no neuronales como las plaquetas, las células sanguíneas, los mastocitos, las células endoteliales, los fibroblastos, las células de Schwann e incluso por las células inflamatorias. Los mediadores inflamatorios también pueden producir dolor al sensibilizar los nociceptores (Millan 1999). Las células residentes, incluyendo las células dendríticas, los macrófagos, los linfocitos y los mastocitos son componentes del tejido que tras reconocer algún estimulo inflamatorio, liberan

factores inflamatorios que estimulan la cascada de citocinas. Existen evidencias de que las citocinas proinflamatorias (ej. IL-1β, TNF-α) y quimiocinas (ej. MCP-1) pueden modular directamente la actividad neuronal en distintos tipos neuronales en el sistema nervioso central y periférico (Figura 8) (Besson 1997, Besson 1999, Coggeshall & Carlton 1997, Dray *et al.* 1994, Guirimand & Le Bars 1996, Millan 1999).

3.2. Receptores nicotínicos, dolor e inflamación.

Desde que en 1992 John Daly y sus colaboradores consiguieran aislar la epibatidina de la piel de una rana del amazonas (*Epipedobates tricolor*) y consiguieran ver que tenía un potente efecto antinociceptivo en la prueba de la placa caliente (200 veces más potente que la morfina), son muchos los estudios realizados con epibatidina en distintas pruebas de dolor. Así, la epibatidina tiene efecto antinociceptivo en la prueba de la retirada de la cola (Boyce *et al.* 2000) y en la de formalina tanto en la versión de la pata (Curzon *et al.* 1998) como inyectada en la región orofacial (Gilbert *et al.* 2001). Además, no solo ha mostrado tener efecto antinociceptivo en modelos agudos de dolor sino también en modelos crónicos (Damaj *et al.* 1998, Rashid & Ueda 2002). Y, no sólo tiene efecto antinociceptivo sino que además ha mostrado efectos anti-hiperalgésicos y anti-alodínicos inducidos por inyección de capsaicina (Lawand *et al.* 1999). En todos estos modelos, el antagonista nicotínico mecamilamina revirtió los efectos antinociceptivos ocasionados por la epibatidina, demostrando así que su efecto está mediado por activación de los nAChRs. El problema del uso de la epibatidina como agente antinociceptivo es que las concentraciones a las que tiene efecto antinociceptivo están cercanas a las concentraciones en las que tiene un efecto tóxico. Por estas razones, las empresas farmacéuticas están muy interesadas en el desarrollo de análogos de epibatidina que, teniendo igual potencia analgésica, no tengan tanta toxicidad como la epibatidina.

La epibatidina no es el único agonista nicotínico que ha mostrado tener efectos antinociceptivos en modelos de dolor. Por ejemplo, la nicotina (Puttfarcken *et al.* 1997, Cepeda-Benito *et al.* 1998, Matsumoto *et al.* 2007) y la colina (Wang *et al.* 2005, Matsumoto et al. 2007) también tienen un efecto antinociceptivo en distintos modelos de dolor. De los distintos nAChR el que ha adquirido más importancia, sobre todo por el efecto antinociceptivo a nivel central, es el $\alpha4\beta2$. Este subtipo de receptor puede modular la liberación de distintos neurotransmisores en la médula espinal (acetilcolina, noradrenalina y serotonina) principalmente a través de la activación de rutas inhibidoras descendentes (Rueter *et al.*

2000). Aunque, los subtipos α7, α5, α9 y α10 están ganando importancia.

El hecho de que las células del sistema inmune, las células derivadas de la médula ósea (linfoides y mieloides) (de Jonge & Ulloa 2007) y la microglía (Shytle *et al.* 2004), expresen gran cantidad de nAChR, hizo pensar que estos receptores podrían desempeñar un papel importante en la inflamación. Las implicaciones funcionales de estos receptores se resolvieron cuando se demostró que la ACh era capaz de controlar la producción de citocinas pro-inflamatorias en macrófagos (Borovikova *et al.* 2000). Además, se demostró que el efecto estaba mediado por los nAChR ya que esta inhibición era más potente cuando se utilizaba nicotina (Wang et al. 2003). En este último trabajo se demostró, además, que la subunidad α7 era crucial en el efecto anti-inflamatorio producido por la señalización del nervio vago. Este efecto se ha corroborado en otro trabajo, donde el efecto anti-inflamatorio de la nicotina se revirtió por antagonistas selectivos del receptor α7 (Ulloa 2005). Este efecto anti-inflamatorio de los agonistas nicotínicos no solo se ha visto en sistema nervioso periférico, sino también en sistema nervioso central. Tanto la acetilcolina como la nicotina son capaces de inhibir la liberación de TNF-α inducida por inyección de LPS en cultivos de microglía de ratón. Además este efecto está mediado por el receptor nicotínico α7 ya que la α-bungarotoxina, un bloqueante selectivo de estos receptores, lo revierte (Shytle et al. 2004).

4. RUTAS INTRACELULARES DE SEÑALIZACIÓN ACTIVADAS POR LOS RECEPTORES NICOTÍNICOS

La nicotina y otros agonistas o moduladores alostéricos de los nAChR han mostrado neuroprotección en varios modelos de toxicidad neuronal, tanto *in vitro* como *in vivo* (O'Neill *et al.* 2002). Aunque las rutas intracelulares que median estos efectos neuroprotectores no son totalmente conocidas, lo que sí se sabe bien es que la neuroprotección es Ca^{2+} dependiente (Donnelly-Roberts *et al.* 1996, Dajas-Bailador *et al.* 2000, Ferchmin *et al.* 2003) y que no implica el bloqueo de los receptores de glutamato (Dajas-Bailador et al. 2000, Ferchmin et al. 2003, Prendergast *et al.* 2001).

Esta entrada de calcio a través del receptor es la que desencadena el inicio de la activación de distintas quinasas con el objetivo de proteger a la célula. De entre estas quinasas primarias cabe destacar el papel de la proteína quinasa c (PKC), proteína quinasa A (PKA), Ca^{2+}/calmodulina quinasa-II (CAMK-II) y fosfoinositol-3-quinasa (PI3K). La PKC y la PKA fosforilan residuos de treonina o serina, modificando la actividad

enzimática de manera específica en cada tipo celular, ejerciendo así efectos sobre el metabolismo. Existen además otros mecanismos menos frecuentes de transferencia de señal que, por ejemplo, afectan a moléculas de membranas tales como la fosfatidilcolina. Otro mecanismo de transducción, a través de la activación de cascadas de quinasas, implica la fosforilación de residuos de tirosina, serina o treonina y tiene lugar en los dominios citoplasmáticos de algunos receptores de membrana; especialmente en receptores para factores de crecimiento. Este sistema es importante en el caso del receptor de insulina, el receptor del IGF (del inglés "insulin growth factor"), hormona de crecimiento (GH) y prolactina (PRL) así como de factores de crecimiento y productos de ciertos oncogenes (PDGF; EGF; FDGF).

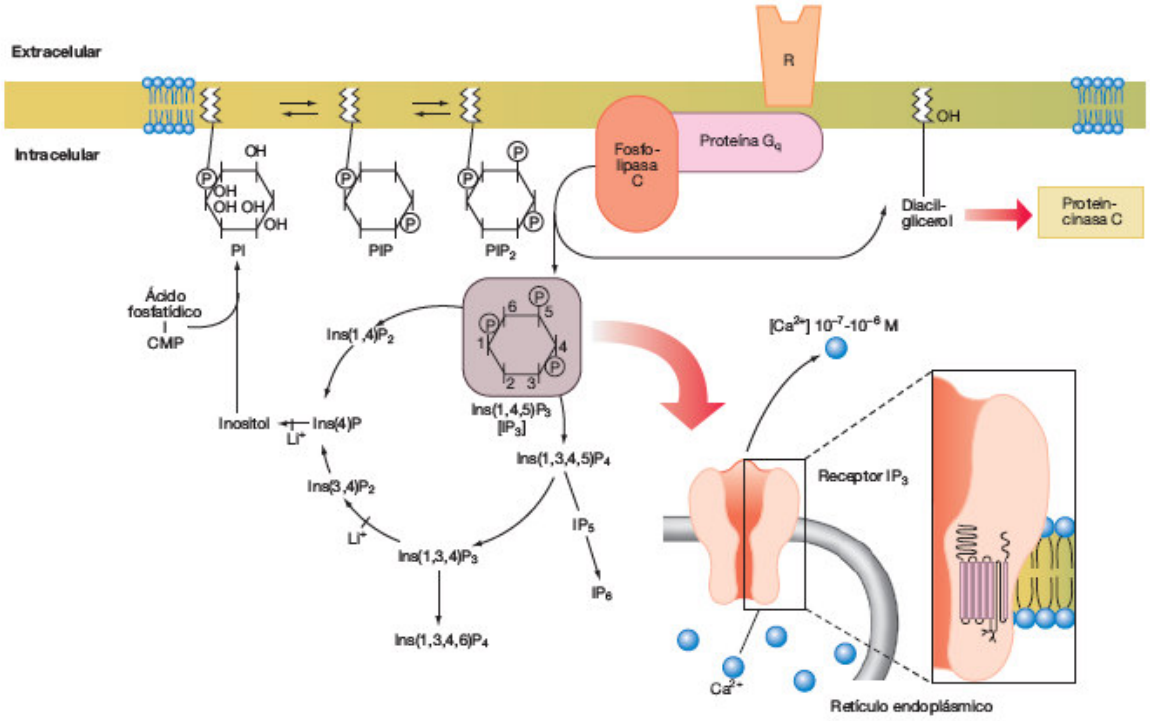

Figura 9. Ciclo del fosfatidilinositol y su papel en la activación de PKC

Para la activación de la PKC es necesaria la activación de la fosfolipasa C que cataliza la hidrólisis de fosfatidilinositol-4,5-bifosfato (PIP2) para formar dos segundos mensajeros, diacilglicerol (DAG) e inositol-1,4,5-trisfosfato (IP3). El IP3 difunde hacia el citoplasma y se une al receptor de IP3 en la membrana del retículo endoplásmico y produce la liberación de calcio al citosol. Este calcio provoca la traslocación de PKC hacia el lado citoplasmático de la membrana lipídica,

uniéndose a residuos de fosfatidilserina; una vez ahí, se activa por la combinación de calcio, el DAG y la presencia de este fosfolípido de membrana (Figura 9). Una vez activa es capaz de fosforilar proteínas o quinasas específicas del citosol, p. ej.: la quinasa regulada por señales extracelulares ERK 1/2 (del inglés, "extracelular signal regulated kinase 1/2"). La activación de PKC a través de los nAChR se ha relacionado con la citoprotección, ya que los inhibidores selectivos de esta proteína revierten la protección que produce la nicotina (Jin *et al.* 2004).

Por otro lado, la PKA se activa por la producción de AMP cíclico, secundaria a la activación de la adenilato ciclasa de la membrana. La activación de PKA también fosforila proteínas específicas relacionadas con la supervivencia celular, tales como la proteína proapoptótica Bad, inhibiendo su efecto proapoptótico (Jin et al. 2004) (Figura 10).

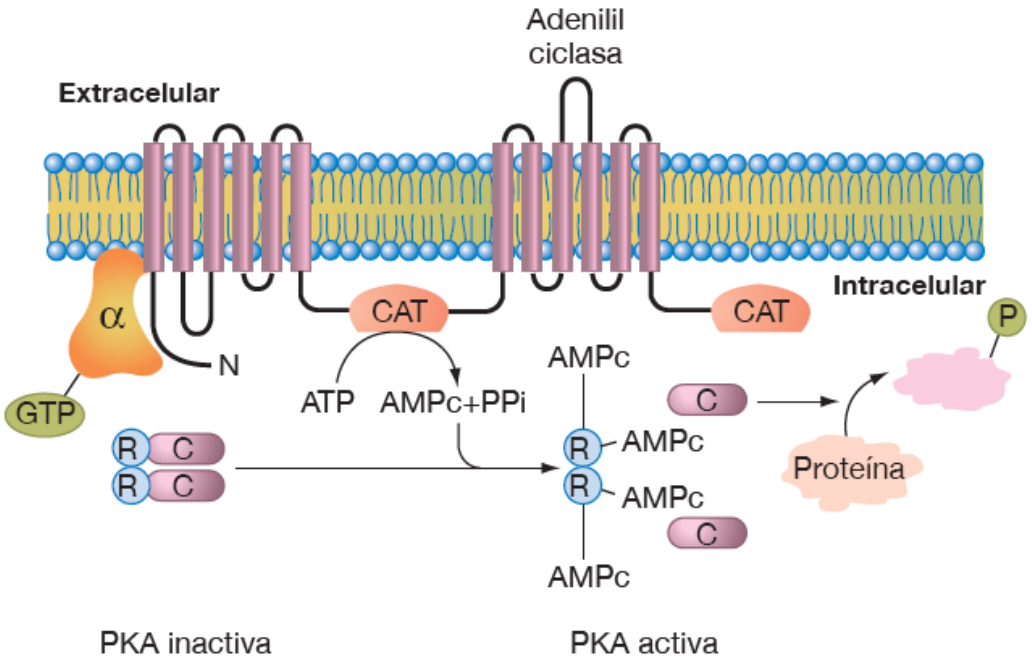

Figura 10. Estructura de adenilciclasa y su acción sobre PKA. CAT: dominio catalítico de la adenil ciclasa; C: unidad catalítica de PKA; R: unidad reguladora de PKA.

Las dos quinasas más importantes y que se relacionan con efectos neuroprotectores en distintos modelos tanto *in vitro* como *in vivo* son la proteína quinasa B o Akt y la ERK 1/2. La primera de ellas, Akt, se activa cuando la quinasa PI3K transforma el PIP2 en fosfoinositol-3, 4, 5-trifosfato (PIP3), que es capaz de reclutar por una parte a Akt, y, por otra, a PDK1 (del inglés "phosphoinositide-dependent kinase

1") (Franke *et al.* 1997). En estas condiciones, la PDK1 fosforila a Akt en 2 residuos (Thr 308 y Ser 473) y la activa. Una vez activada, Akt es capaz de fosforilar a múltiples proteínas en residuos de serina o treonina, asumiendo un papel clave en distintos procesos celulares tales como el metabolismo de la glucosa, la proliferación celular o la supervivencia celular. De las múltiples proteínas que Akt es capaz de fosforilar en relación con la supervivencia celular, cabe destacar a BAD, caspasa-9 y GSK-3β.

Otra quinasa que se relaciona con efectos neuroprotectores es Erk1/2. Esta quinasa pertenece a la familia de las MAPKs (del inglés "mitogen-activated protein kinases"). Para la activación de Erk1/2 es necesaria la activación consecutiva de otras 3 quinasas de la ruta de señalización de las MAPKs: Ras-Raf-MEK1/2. Una vez activada, la MEK1/2 (del inglés "MAP/ERK kinase 1/2"), fosforila los residuos de treonina y tirosina en la secuencia Thr-Glu-Tyr de Erk1/2 y la activa. Una vez activada, Erk1/2 fosforila muchos sustratos, incluyendo factores de transcripción como Elk1 o c-Myc o quinasas como la RS6K (del inglés "Ribosomal S6 kinase"). De esta manera se activan genes de respuesta temprana, relacionados sobre todo con la proliferación celular y la supervivencia celular.

Así, la activación de distintas rutas de señalización es necesaria para la prevención de la muerte neuronal, aunque no existe consenso sobre qué ruta intracelular es la que predomina.

5. HEMO OXIGENASA-1, NEUROPROTECCIÓN Y DOLOR.

La hemoxigenasa es una enzima limitante en la degradación del grupo pro-oxidante hemo en tres productos distintos: hierro, CO y biliverdina. La biliverdinase convierte en bilirrubina mediante la intervención de la biliverdina reductasa (Ryter *et al.* 2006). Recientemente, han surgido un gran número de evidencias que mencionan que la HO es importante en otras funciones fisiológicas como son la citoprotección en modelos de estrés celular (Kim *et al.* 2005), la inflamación (Poss & Tonegawa 1997) o el dolor (Li & Clark 2003, Li & Clark 2000, Steiner *et al.* 2001).

Existen dos isoformas genéticamente diferentes de la HO: la HO-1 que es inducible, y la HO-2 que es constitutiva (Ryter et al. 2006). La HO-1 se expresa en niveles que están entre bajos y no detectables en condiciones basales, en tejidos que no están implicados en el metabolismo de eritrocitos o de la hemoglobina, pero responde rápidamente a una activación transcripcional por diversos estímulos químicos y físicos (Ryter et al. 2006). El sustrato natural de la HO-1, el grupo hemo, que sirve de señal de alerta

ante una lesión tisular; cuando se libera localmente, puede funcionar como un desencadenante natural del proceso inflamatorio. La hemina, la forma oxidada del grupo hemo, induce de manera dependiente de dosis a la HO-1, con un aumento máximo de cerca de 20 veces. Por otro lado, la HO-1 inhibe la inflamación, disminuyendo la expresión de moléculas de adhesión y la adhesión consiguiente de leucocitos; constituye éste la base de un mecanismo tardío de autorregulación de la inflamación (Wagener *et al.* 2003).

El factor de transcripción Nrf2 puede inducir la expresión de la HO-1 por el grupo hemo (Alam *et al.* 2003). Curiosamente el Nfr2 también está implicado en el proceso de curación de heridas, siendo activado en fases tempranas de la herida por los ROS allí generados (Braun *et al.* 2002). La protoporfirina de cobalto (CoPP), que tiene estructura similar a la de la hemina, pero tiene cobalto como grupo prostético en vez de hierro, también puede inducir la expresión de HO-1 mediante la activación del factor de transcripción Nrf2 (Shan *et al.* 2006).

Hay muchos estudios que relacionan la HO-1 con efectos protectores, no sólo a nivel cerebral (Diaz *et al.* 2005, Vargas *et al.* 2005, Hwang & Jeong 2008), sino también en enfermedades vasculares (Chung *et al.* 2008), en modelos de inflamación aguda (Takahashi *et al.* 2007), en modelos de hepatotoxicidad (Farombi & Surh 2006), o en modelos de isquemia renal (Takahashi *et al.* 2004).

Existen pocos estudios que relacionen la HO-1 y las señales nociceptivas. A pesar de que no existen evidencias directas de la participación de la HO-1 en la nocicepción, se ha sugerido que el CO producido por la HO podría limitar la hiperalgesia inflamatoria en la pata de ratas y que tal efecto estaría mediado por la enzima guanilato ciclasa soluble y la producción de GMPc (Steiner et al. 2001).

La HO-2 se ha implicado en la transmisión de la señal nociceptiva en la médula espinal. Experimentos con doble tinción han demostrado que un alto porcentaje de núcleos positivos para la proteína Fos, después de la administración de formalina, se localizaban dentro de células positivas para HO-2 (Li & Clark 2001a). La HO-2 participa en el dolor inflamatorio y neuropático (Li & Clark 2003) y la producción de CO mediada por HO-2 parece estar relacionada con la transmisión glutamatérgica (Li & Clark 2002). Los ratones con ablación del gen que codifica para HO-2 sienten menos dolor en el modelo de la formalina en la primera y segunda fases de la prueba (Li *et al.* 2004). Además, su inhibición en la médula espinal mejora el efecto de la morfina (Li & Clark 2001b); por tanto, se ha sugerido que la HO-2 puede estar implicada en el proceso de tolerancia a los opiáceos (Liang *et al.* 2003).

III. OBJETIVOS

OBJETIVOS DE ESTA TESIS DOCTORAL

1. Evaluar el efecto neuroprotector de galantamina y memantina en el modelo de privación de oxígeno y glucosa en rodajas de hipocampo de rata.

2. Estudiar los posibles mecanismos implicados en el efecto neuroprotector de la galantamina, en concreto, la vía de señalización de PI3K/Akt e iNOS.

3. Evaluar si el efecto neuroprotector mediado por nicotina frente a la privación de oxígeno y glucosa está relacionado con algún subtipo concreto de receptor nicotínico.

4. Evaluar la implicación de la vía de señalización de Erk1/2 y la hemo oxigenasa-1 en el efecto neuroprotector de la epibatidina en el modelo de estrés oxidativo.

5. Evaluar el posible efecto analgésico de la inducción de hemo oxigenasa-1 en el modelo de inyección de formalina en la pata.

6. Estudiar si la inducción de hemo oxigenasa-1 por epibatidina tiene un efecto antinociceptivo en el modelo de inyección de formalina en la pata.

IV. RESULTADOS

1. NEUROPROTECCIÓN

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Galantamine and memantine produce different degrees of neuroprotection in rat hippocampal slices subjected to oxygen–glucose deprivation

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Abstract

Recent clinical trials have shown that galantamine is efficacious in the treatment of mild to moderate Alzheimer's and vascular dementia, and memantine in severe stages of these diseases. Hence, the hypothesis that these two drugs might exert different degrees of neuroprotection has been tested. Rat hippocampal slices were subjected to oxygen and glucose deprivation (OGD) and to a re-oxygenation period. Neuronal damage was monitored using the lactate dehydrogenase (LDH) released into the Krebs-bicarbonate medium as an indicator. Galantamine, a mild acetylcholinesterase (AChE) blocker and nicotinic receptor modulator, given 30 min before and during OGD plus re-oxygenation (1, 2 and 3 h) significantly reduced LDH release by around 50%. Galantamine 5 μM reduced LDH release significantly during the re-oxygenation period while at 15 μM it afforded significant reduction of LDH release both during OGD and re-oxygenation. Memantine, a reversible blocker of NMDA receptors, at 10 μM only significantly reduced (40%) LDH release after 3 h re-oxygenation. The classical NMDA blocker MK-801 reduced LDH released around 40% at 1 μM at all re-oxygenation times studied. These data indicate that galantamine has a neuroprotective window against anoxia wider than memantine. Whether these differences can be clinically relevant remain to be studied in appropriate clinical trials.

Keywords: Oxygen–glucose deprivation; Galantamine; Memantine; Hippocampal slices

Recent clinical trials show therapeutic benefits in cognition, functional and behavioral symptoms exerted by galantamine and memantine in dementia of the Alzheimer's and vascular types. However, while galantamine has shown efficacy in patients at mild-to-moderate stages of these diseases [17,22], memantine shows efficacy in advanced stages [6]. Therefore, the question arises to whether these clinical differences are due to their different mechanisms of action and/or to different degrees of neuroprotection exerted by these two drugs. Galantamine is a mild inhibitor of acetylcholinesterase (AChE) and an allosteric potentiator of neuronal nicotinic receptors [12], whereas memantine is a non-competitive, voltage-dependent, reversible blocker of NMDA receptors for glutamate [15]. Both compounds exhibit neuroprotective effects in vitro [3,15] as well as in vivo neuronal death models [4,15]. However, a study comparing simultaneously the neuroprotective effects of galantamine and memantine in the same model is lacking. Therefore, we decided to compare the neuroprotective effect of galantamine and memantine in an acute model of neuronal damage exerted by oxygen and glucose deprivation (OGD) in the rat hippocampal slice.

Abbreviations: OGD, oxygen and glucose deprivation; LDH, lactate dehydrogenase; AChE, acetylcholinesterase; i.p., intraperitoneal; mOD, mean optical density; APL, allosteric potentiator ligand; TRPM, Transition Receptor Potential Cation Channel

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All experiments were performed using the brain of adult male Sprague–Dawley rats (275–325 g) from a colony of our animal quarters; they were performed following the rules of the Ethical Committee for the Care and Use of Animals in Research, of our medical school. Rats were deeply anesthetized with sodium pentobarbital (60 mg/kg i.p.), decapitated, and each brain was rapidly removed from the skull and placed into ice-cold Krebs bicarbonate buffer, dissection buffer (pH 7.4), containing (in mM): NaCl 120, KCl 2, $CaCl_2$ 0.5, $NaHCO_3$ 26, $MgSO_4$ 10, KH_2PO_4 1.18, glucose 11 and sucrose 200 [13]. All chamber solutions were pre-bubbled with either 95% O_2/5% CO_2 or 95% N_2/5% CO_2 gas mixtures, for at least 45 min before slice immersion, to ensure O_2 saturation or O_2 removal as desired. The hippocampi were quickly dissected, glued down leaning vertically against agar blocks in a small chamber, submerged in cold, oxygenated dissection buffer and sectioned in transverse slices of 350 m thick (dissection period) using a vibratome (Leica; Heidelberg, Germany). A basal and OGD group was included in all experiments. A maximum of three drug concentrations was tested in each experiment. Immediately after vibratome sectioning, the slices were transferred to vials of sucrose-free dissection buffer, bubbled with 95% O_2/5% CO_2 in a water bath at room temperature for 60 min to recover from slicing trauma before starting the experiments (equilibration period).

After an initial preincubation period of 30 min, the slices corresponding to the basal group were incubated 60 min in a normal Krebs solution, having the following composition (in mM): NaCl 120, KCl 2, $CaCl_2$ 2, $NaHCO_3$ 26, $MgSO_4$ 1.19, KH_2PO_4 1.18 and glucose 11; this solution was equilibrated with 95% O_2/5% CO_2. Oxygen and glucose deprivation was induced by incubating the slices for a 60 min period in a glucose-free Krebs solution, equilibrated with a 95% N_2/5% CO_2 gas mixture; glucose was replaced with 2-deoxyglucose. After this OGD period, the slices were returned back to an oxygenated normal Krebs solution containing glucose (re-oxygenation period). These experiments were performed at 36.5 °C. When used, memantine (1, 3, and 10 μM) and galantamine hydrobromide (0.3, 1, 5 and 15 μM) were added to the OGD slices during the 30 min of preincubation period and remained there during the OGD and re-oxygenation period (see protocol in Fig. 1A).

The viability of hippocampal slices was monitored by measuring the activity of the lactate dehydrogenase (LDH) released into the incubation media [9]. Samples of this solution were taken at the end of the preincubation period and at times 0, 60, 120 and 180 min of the re-oxygenation period (see protocol in Fig. 1). LDH activity was measured spectrophotometrically at 490–600 nm, using a microplate reader (Labsystems iEMS reader MF). The remaining LDH was obtained by incubating the slices with 1% Triton X-100 at the end of the experiment, for 30 min; then, the samples were centrifuged and an aliquot from the supernatant was taken to measure the intracellular LDH. LDH levels remaining in the basal and OGD-slices after 3 h of reperfusion were high and

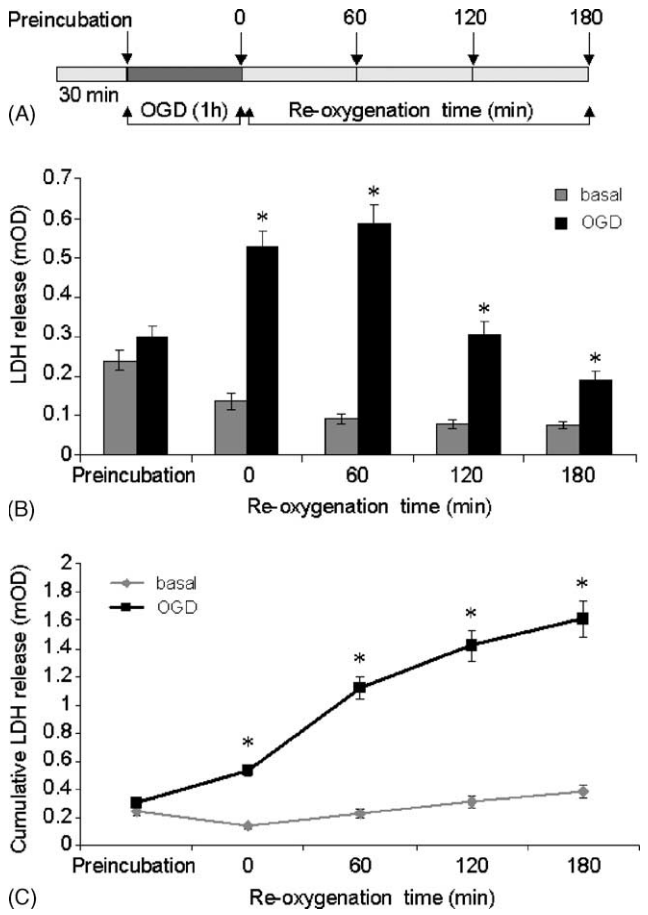

Fig. 1. Neuronal damage measured as LDH released into the medium, caused by oxygen and glucose deprivation (OGD) followed by re-oxygenation plus glucose reintroduction in rat hippocampal slices. Panel A shows the protocol (see methods). Panel B shows the basal LDH release and the release of LDH evoked by OGD plus re-oxygenation, in each experimental period (abscissa). Panel C shows the cumulative values of LDH released into the media (basal and OGD), calculated by adding the individual values of each collection period of incubation period from OGD to 3 h re-oxygenation, in each experiment. Data of LDH activity in (B) and (C) are expressed as mOD and they are means + S.E.M. of 29 experiments. $^*P < 0.05$, compared with basal.

not significantly different from each other (4.008 + 0.038 versus 4.002 + 0.058 mean optical density (mOD) in basal and OGD-slices, respectively, $n = 8$, $P < 0.05$). Thus, only the LDH released into the medium was measured (see results).

MK-801 (1 μM), memantine (1, 3, and 10 μM) and galantamine hydrobromide (0.3, 1, 5 and 15 μM) were added to the OGD slices during the 30 min of preincubation period and remained during the OGD and re-oxygenation period. MK-801 was obtained from Tocris (Biogen Científica, Spain), memantine from Sigma (Aldrich, Spain) and galantamine hydrobromide from Janssen (Beerse, Belgium). They were dissolved in saline just before each experiment.

LDH efflux was expressed as the LDH activity present in the incubation solution measured as mOD. Data are represented as means + S.E.M. Differences between treatments,

as a function of drug concentration and re-oxygenation time, were estimated by applying the analysis of variance (ANOVA) and Fisher's test. Differences were considered to be statistically different when $P = 0.05$.

In our present study, LDH release in basal conditions accounted for less than 25% of the maximum LDH released after OGD followed by 3 h reperfusion. Thus, we considered that there was a wide window to explore neuronal damage evoked by OGD/re-oxygenation, and the effects of galantamine and memantine on such damage.

These slices were subjected to the protocol of sequential incubations shown in Fig. 1A. Those incubations scarcely affected the total tissue LDH activity measured at the end of the experiment; this suggests that the overall tissue suffered little damage during the 4 h and 30 min period of the experiment. Hence, the total LDH activity remaining in the tissues at the end of the experiment was not measured in subsequent experiments. Only the LDH released into the different incubation media was taken as an indicator of neuronal tissue damage. It is likely that the LDH released into the media during OGD and re-oxygenation mostly came from the pyramidal neuron layer of the CA1 area of the hippocampus, which is known to be particularly sensitive to anoxia [19]. The basal LDH released into the incubation media from slices not subjected to OGD decreased with time (gray columns of Fig. 1B). LDH released rose as much as fourfold above basal after the 1 h OGD period (0 h of re-oxygenation in Fig. 1B). LDH released augmented further to sixfold above basal, during the first hour of re-oxygenation. Then, it gradually declined at 2 and 3 h re-oxygenation (black columns of Fig. 1B). The increments above basal LDH released into the media, elicited by OGD, were better seen if added in a cumulative manner from OGD to 3 h re-oxygenation, as Fig. 1C shows. Thus, the effects of drugs on LDH released were plotted in this cumulative manner in all subsequent experiments.

To study the effects of galantamine on the release of LDH from hippocampal slices subjected to OGD and re-oxygenation, the protocol performed was similar to that described in Fig. 1A. Every experiment included slices to measure the basal and the OGD plus re-oxygenation-induced LDH release; parallel slices contained various concentrations of galantamine, which were present during 30 min of the equilibration period, as well as throughout the OGD and re-oxygenation periods. Note that 0.3 and 1 μM galantamine slightly enhanced LDH release at 2 and 3 h re-oxygenation; however, this increase was not statistically significant. In contrast, 5 μM galantamine significantly reduced LDH release at 60, 120 and 180 min of re-oxygenation time; the reduction accounted for 45% after 1 h re-oxygenation, 51% after 2 h re-oxygenation, and 56% after 3 h re-oxygenation. Fifteen micromolar galantamine reduced LDH release by 56, 47, 50 and 54% at 0, 1, 2, 3 h re-oxygenation times, respectively (Fig. 2).

Experiments similar to those of galantamine were performed with various concentrations of memantine. Fig. 3A

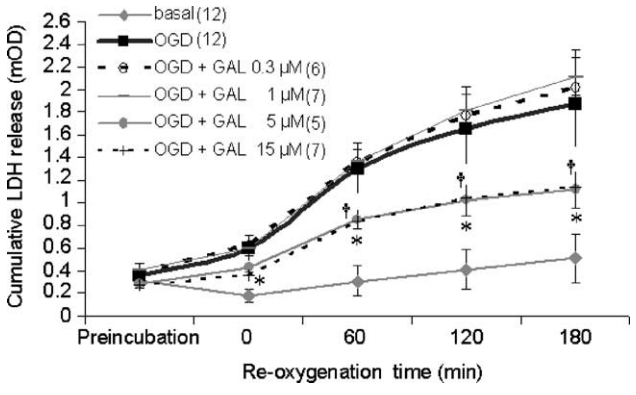

Fig. 2. Effects of galantamine (GAL) on the release of LDH from hippocampal slices subjected to OGD and re-oxygenation. Slices were run in parallel to measure the LDH released in basal or OGD conditions, in the absence or the presence of the indicated concentration of galantamine. Different concentrations of the drugs were tested in different experiments. LDH release is cumulatively expressed in the abscissa (see Fig. 1). Data are means + S.E.M. of the number of experiments shown in parentheses. $^{*,\dagger}P < 0.05$, compared with OGD.

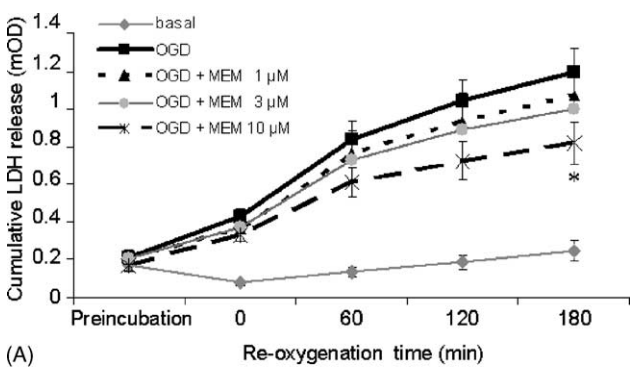

Fig. 3. Effects of memantine (MEM) and MK-801 on the release of LDH from hippocampal slices subjected to OGD. Slices were run in parallel to measure the LDH released in basal or OGD conditions, in the absence or the presence of the indicated concentrations of memantine (panel A) or MK-801 (panel B). Different concentrations of the drugs were tested in different experiments. Data correspond to means + S.E.M. of eight experiments for the dose–response curve of memantine (A) and nine experiments for MK-801 (B). $^{*}P < 0.05$, compared with OGD.

shows that 1 and 3 μM of memantine reduced the release of LDH evoked by OGD plus re-oxygenation by 13–20%, but this reduction was not statistically significant. At 10 μM, memantine reduced LDH release by 28–37% at 0–2 h re-oxygenation and by 40% at 3 h re-oxygenation. We used theNMDA receptor blocker MK-801 for comparative purposes. At 1 μM, MK-801 significantly reduced LDH release at all times of re-oxygenation, reaching by 42% after OGD and 3 h re-oxygenation (Fig. 3). No further reduction was seen at higher concentrations of 10 and 30 μM (not shown). This is the first study showing that an inhibitor of AChE, i.e. galantamine, has neuroprotective effects against neuronal damage caused by anoxia, in hippocampal slices. Furthermore, this is also the first study which compares in the same preparation (i.e. rat hippocampal slices subjected to OGD) the neuroprotective effects of galantamine and memantine, two drugs recently introduced in the clinic to treat patients with Alzheimer's and vascular type of dementia [11,18]. We have found in this study that galantamine is more efficacious than memantine as a neuroprotective agent which, considering their mechanism of action, was an unexpected result. LDH release was originally used to measure neuronal cell death occurring via necrosis [9], although subsequent studies suggested that LDH release was also a good marker of apoptotic neuronal death [10]. More recent studies validated LDH release as an adequate index of necrotic tissue damage in brain slice preparations [5,13]. Although we tried other markers of neuronal death (i.e. MTT, propidium iodide), we found that LDH gave the highest and more reproducible window of injury.

It is well established that the hypoxia following a transient cerebral ischemic insult causes excessive accumulation of glutamate in the synapse, with the activation of the high Ca^{2+}-permeable NMDA receptors. This leads to Ca^{2+} overloading and neuronal cell death [21]. Thus, it is ex- pected that non-competitive irreversible (i.e. MK-801) and reversible NMDA receptor blockers (i.e. memantine) should mitigate the neuronal damage due to ischemic–anoxic con- flicts. This has been shown to be the case in a number of in vitro [20] and in vivo models [8,15]. In the present study, we have corroborated the neuroprotective effects of MK-801 and memantine in rat hippocampal slices subjected to anoxia stress. However, we found a surprising finding. This neuroprotective effect was mild and did not follow a clear concentration-dependence. Thus, 1 μM MK-801 afforded 42% of neuroprotection, while 10 μM did not offer further protection. The memantine case was even more striking, since 1–3 μM showed no protection and 10 μM exhibited only some protection after 3 h re-oxygenation; no protection was seen at 0–2 h re-oxygenation. This contrasts with the protective effects of memantine observed in other models of neuronal damage in this concentration range. For instance, in neuronal cultures exposed to hypoxic conditions, memantine exhibited clear neuroprotection at 1 μM [20]. It is possible that in our acute model of "cerebral ischemia," other mechanisms intervene in addition to the excessive activation of NMDA receptors by endogenously released glutamate. This is substantiated by the striking and powerful neuroprotective effects afforded by galantamine.

Galantamine has a dual mechanism of action that might explain its therapeutic benefits on cognition, functional activities and behavior in patients with dementia of the Alzheimer's and vascular types [17,22]. On the one hand, galantamine behaves as a mild inhibitor of AChE; on the other, it acts as an allosteric potentiator ligand (APL) at presynaptic nicotinic receptors [12]. We have recently shown that submicromolar concentrations of galantamine affords protection against cell damage in cultures of human neuroblastoma cells and bovine chromaffin cells, exposed to apoptotic agents such as thapsigargin or β-amyloid. Such protection was associated to α7 nicotinic receptors and the induction of the expression of the anti-apoptotic protein Bcl-2, and required 24–48 h preincubation periods with galantamine [3]. This could be explained by the APL effect of galantamine, which only appears at concentrations below or around 1 μM. In our present study, however, the neuroprotective effects of galantamine were visible rather at higher (10- to 20-fold) concentrations and developed in the time lapse of 4–5 h. Neuroprotection was similar at 5 and 15 μM, concentrations at which the APL effect of galantamine turns into a nicotinic receptor blockade [12]. Thus, its pronounced neuroprotective effects at 5–15 μM must be attributed to other galantamine effects, i.e. the inhibition of AChE. In fact, galantamine is a weak inhibitor of this enzyme, exhibiting an IC_{50} of about 3 μM [23], well in the range where the drug exerts its drastic neuroprotective action. It is also plausible that the inhibition of small-conductance Ca^{2+}-activated K^+ channels, recently discovered in our laboratory [2], might also contribute to its neuroprotective action. A new challenging hypothesis has been raised to explain why many compounds that show neuroprotective efficacy in animal models of focal cerebral ischemia, do not afford protection in clinical trials done in stroke patients. The hypothesis implies that after prolonged ischemic episodes (30 min or more) a "Transition Receptor Potential Cation Channel" (TRPM) opens following Ca^{2+} entry, cell Ca^{2+} overloading and neuronal cell death. This pore is a Ca^{2+} entry pathway that is not blocked by NMDA or AMPA receptor blockers [1]. Whether in our experimental conditions (i.e. 60 min OGD) this pore is contributing to neuronal damage is uncertain. But one possibility is that galantamine might be blocking such "TRPM."

It is interesting that galantamine and memantine show therapeutic benefit in both patients with dementia of Alzheimer's or vascular types, suggesting a common pathogenic mechanism. This may be linked to neuronal Ca^{2+} overloading and excess Ca^{2+} accumulation into mitochondria, leading to free radical overproduction and cell death. A Ca^{2+} dyshomeostasis causing a loss of Ca^{2+} from the endoplasmic reticulum, leading to apoptosis, has been implicated as an important metabolic cross-road in the pathogenesis of Alzheimer's disease [16]. In the case of

vascular dementia, the neuronal Ca^{2+} overload may come from excessive glutamate acting on NMDA receptors, as stated above. Both, in Alzheimer's disease [7] and during brain ischemic conditions [14] glutamatergic as well as cholinergic neurotransmission are affected. On other hand, free radicals seem to be involved in neurodegenerative process; in this context, it is interesting that a recent study reports the antioxidant properties of galantamine [24].

In conclusion, whatever the mechanism(s) involved, it seems clear that in rat hippocampal slices subjected to OGD and re-oxygenation, galantamine has a neuroprotection window wider than memantine. Our findings may orient the design of new comparative clinical trials between galantamine and memantine, in patients with Alzheimer's and/or vascular dementia, at different stages of the disease and with different dose ranges. They also suggest a possible neuroprotective role of galantamine in acute cerebral ischemia. We are presently testing this hypothesis in animal models of focal and global cerebral ischemia. If these experiments show that galantamine also exhibits neuroprotective properties in these in vivo models, a clinical trial in stroke patients may be indicated.

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Role of PI3K/Akt-GSK3β-BAD pathway and iNOS in the neuroprotective effect of galantamine in hippocampal slices subjected to oxygen and glucose deprivation

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Background and purposes: Galantamine is a drug currently used to treat Alzheimer´s disease, however, concomitant ischemic brain injury in these patients can contribute to accelerate their cognitive deficit. We previously showed that galantamine could afford neuroprotection in *in vitro* and *in vivo* ischemia models. Following this research line, the current study was planned to investigate the intracellular signaling pathways implicated in the protective effect of galantamine in an ischemia-reperfusion model.
Experimental approach: We used rat hippocampal slices subjected to 15 min oxygen and glucose deprivation (OGD) followed by 60 min reoxygenation.
Key results: In this model, cell death measured as the ratio of propidium iodide/Hoechst fluorescence duplicated with respect to control slices. In slices treated with galantamine, cell death was reduced to almost control levels. Such neuroprotection was accompanied by a significant reduction in free radical production measured as fluorescence of DFCDA. Furthermore, galantamine treatment reduced by 53-58% NO production caused by OGD-reoxygenation; this effect correlated with a significant reduction of iNOS. OGD-reoxygenation reduced phosphorylation of Akt, significantly reduced GSK-3β inhibition by dephosphorylation of it Ser-9 and also decreased p-Bad; galantamine treatment normalized all these values. The neuroprotective effects of galantamine were related to its action on nicotinic receptors and the PI3K/Akt pathway since mecamylamine and LY294002 reverted its protective effect.
Conclusions and implications: Galantamine protected hippocampal neurons subjected to OGD-reoxygenation by reducing NO production via inhibition of iNOS and inhibiting GSK-3β and inactivating Bad via PI3K-Akt. Therefore, galantamine could benefit patients undergoing brain ischemia.

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Introduction

We have designed this study to gain information on how galantamine exerts its neuroprotective action in an ischemia-reperfusion model. Ischemic brain injury secondary to cardiovascular disease is a common cause of dementia and cognitive decline in the elderly. Cerebrovascular disease also contributes to cognitive loss in Alzheimer's disease. Galantamine is an acetylcholinesterase inhibitor currently used to treat mild to moderate Alzheimer´s disease patients. Controlled clinical trials with galantamine in patients with vascular disease, as well as in patients with Alzheimer's plus cerebrovascular disease, have demonstrated improvement in cognition, behavior and activities of daily living (Erkinjuntti et al., 2003). Although galantamine has shown to be neuroprotective by our and other groups in Alzheimer´s disease (Arias et al., 2004; Arias et al., 2005; Takada-Takatori et al., 2006) and brain ischemia (Lorrio et al., 2007; Sobrado et al., 2004) related models, the intracellular mechanism by which galantamine can be affording such protection has not yet been described in a brain ischemia model.

One of the main events during ischemia, and especially during reperfusion, is the generation of free radicals; due to their high reactivity, they provoke damage to lipids, DNA and proteins and, produce neuronal death. They also contribute to the breakdown of the blood-brain barrier and brain edema. One of the radicals elevated after the ischemic insult is nitric oxide (NO). NO can be generated primarily by NO synthases (NOS). There are three isoforms of this enzyme, the endothelial (eNOS) and the neuronal (nNOS) which are constitutively expressed and a third inducible isoform termed iNOS (Moro et al., 2004). iNOS is expressed after ischemia and contributes to cell damage associated to this condition. This is supported by different studies that show that iNOS mRNA, protein and enzymatic activity are expressed in the brain after transient or permanent brain ischemia in rodents (Grandati et al., 1997; Iadecola et al., 1995) and in *in vitro* models of brain ischemia (Cardenas et al., 2000; Moro et al., 1998). iNOS can be regulated by inflammatory cytokines (Galea *et al.*, 1992; Peterson *et al.*, 1994; Simmons *et al.*, 1992), by oxidative stress itself (Melillo et al., 1995) or by glutamate (Cardenas et al., 2000) released after an ischemic episode.

On the other hand, the serine/threonine kinase PI3K/Akt is a key component in survival signaling pathways (Manning *et al.*, 2007). In

the central nervous system, decreased Akt activity has been linked to neuronal death induced by NMDA receptor activation, focal ischemia or hypoxia (Zhao et al., 2005). After activation, Akt phosphorylates target proteins involved in cell growth, metabolism and survival (Manning *et al.*, 2007).

Akt can target glycogen synthase kinase 3 (GSK3), a serine/threonine protein kinase that phosphorylates and thereby inactivates glycogen synthase (Embi et al., 1980; Hemmings et al., 1981; Rylatt et al., 1980). There are two isoforms, GSK-3α and GSK-3β; the latter is particularly abundant in the CNS, and is neuron specific (Leroy *et al.*, 1999). GSK-3β phosphorylates and thereby regulates many important metabolic and signalling proteins, structural proteins and transcription factors (Frame *et al.*, 2001). Inhibition of this enzyme has been shown to be protective against a plethora of neurological insults including cerebral ischemia (Manji et al., 1999; Nonaka et al., 1998), suggesting that an inhibition of GSK-3β improves brain cell survival.

Another target protein for Akt is the pro-apoptotic protein Bad, preventing it from binding to and inactivating Bcl-xL in mitochondria (Datta et al., 1997). In turn Bcl-xL exerts its antiapoptotic effect and contributes to cell survival. In cerebral ischemia, Bad has been shown to be a key molecule regulating the balance between cell survival and cell death signals (Kamada et al., 2007).

Considering the importance free radical oxygen species (ROS) production in inducing neuronal death and the importance of the PI3K/Akt pathway in mediating cell survival by phosphorylation of GSK-3β and Bad during an ischemic insult, we have analysed the participation of these proteins during control conditions and during ischemia alone or in the presence of galantamine. The results of this study will not only afford information on the neuroprotective mechanism of galantamine during an ischemic insult but can also be useful to design new drugs targeting these pathways for stroke patients.

Methods

The fluorescent dyes propidium iodide, Hoechst 33342 and DCFDA were from Invitrogen (Madrid, Spain). Anti-iNOS was purchased from BD Europe Transduction lab (Madrid, Spain), anti-phospho-Ser9-GSK-3β, anti-GSK-3β, anti- phospho-Thr308-Akt, anti-Akt and anti-phospho-Ser136-BAD were from Cell Signaling (Izasa S.A., Barcelona, Spain), anti-β–actin and mecamylamine from Sigma (Madrid, Spain) and LY294002 and galantamine form Tocris (Biogen Científica, Spain).

Animal usage and hippocampal slice preparation

All experiments were performed using adult male Sprague–Dawley rats (275–325 g) from a colony of our animal quarters. The experiments were performed after approval of the protocol by the institutional Ethics Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiments.

We used the protocol described by Egea and co-workers (Sobrado *et al.*, 2004). Rats were quickly decapitated under sodium pentobarbital anesthesia (60 mg/kg, i.p.), forebrains were rapidly removed from the skull and placed into ice-cold Krebs bicarbonate dissection buffer (pH 7.4), containing (in mM): NaCl 120, KCl 2, $CaCl_2$ 0.5, $NaHCO_3$ 26, $MgSO_4$ 10, KH_2PO4 1.18, glucose 11 and sucrose 200. The chamber solutions were pre-bubbled with either 95% O_2/5% CO_2 or 95% N_2/5% CO_2 gas mixtures, for at least 45 min before slice immersion, to ensure O_2 saturation or removal. The hippocampi were quickly dissected, glued down leaning vertically against agar blocks in a small chamber, submerged in cold, oxygenated dissection buffer and sectioned in transverse slices of 200 µm (slices for imaging) or 350 µm (slices for glutamate and nitrates measurements) thick using a vibratome (Leica; Heidelberg, Germany). After an initial preincubation period of 30 min, the slices corresponding to the control group were incubated 15 or 60 min in a Krebs solution with the following composition (in mM): NaCl 120, KCl 2, $CaCl_2$ 2, $NaHCO_3$ 26, $MgSO_4$ 1.19, KH_2PO_4 1.18 and glucose 11; this solution was equilibrated with 95% O_2/5% CO_2. Oxygen and glucose deprivation was induced by incubating the slices for a 15 or 60 min period in a glucose-free Krebs solution, equilibrated with a 95% N_2/5% CO_2 gas mixture; glucose was replaced by 2-deoxyglucose. After this OGD period, the slices were returned back to an oxygenated normal Krebs solution containing glucose (re-oxygenation period) for 1 or 3 h. Experiments were performed at 37 °C. A control and OGD group was included in all experiments.

Quantification of cell death in propidium iodide and Hoechst stained hippocampal slices

At the end of the experiment, the hippocampal slices were loaded with 1 µg/ml PI and Hoechst 33342 in the last 5 min of incubation. Mean PI and Hoechst fluorescence in CA1 and CA3 regions in each slice, after the given treatments, were analyzed. Fluorescence was measured in a fluorescence inverted NIKON eclipse T2000-U microscope. Wavelengths of excitation and emission for PI and Hoechst were 530 or 350, and 580 or 460 nm, respectively. Images were taken at CA1 and CA3 at magnifications of 100X. Fluorescence analysis was performed using the Metamorph programme version 7.0. To calculate cell death, we divided the mean PI fluorescence by the mean Hoechst fluorescence. Data was normalized with respect to control values that were considered as 1.

Measurement of ROS production in hippocampal slices

To measure cellular reactive oxygen species (ROS), we have used the molecular probe H_2DCFDA (Ha et al., 1997) which diffuses through the cell membrane and is hydrolyzed by intracellular esterases to the non-fluorescent form dichlorofluorescein (DCFH). DCFH reacts with intracellular H_2O_2 to form dichlorofluorescin (DCF), a green fluorescent dye. Immediately after vibratome sectioning, 200 µm thick hippocampal slices were loaded with 80 µM H_2DCFDA for 45 min in Krebs solution (120 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, 1.19 mM $MgSO_4$, 1.18 mM KH_2PO_4 and 11 mM glucose). Subsequently, slices were washed twice with Krebs solution and kept for 15 min before the beginning of the experiment. Fluorescence was measured in a fluorescence inverted NIKON eclipse T2000-U microscope. Wavelengths of excitation and emission were 485 and 520 nm, respectively. Images were taken at CA1 and CA3 at magnifications of 100X. Fluorescence analysis was performed using the Metamorph programme version 7.0. Fluorescence in basal conditions was taken as 1 and experimental variables were normalized with respect to this value.

NO_2^- assay

NO release was estimated from the amounts of nitrites (NO_2^-) in the incubation solution. NO_2^- was determined by a colorimetric assay based on the Griess reaction and modified by Marzinzig *et al*. (Marzinzig et al., 1997). Samples of this solution were taken at times 0, 60, 120 and 180 min of the re-oxygenation period and then, measured spectrophotometrically at 540 nm, using a microplate reader (Labsystems iEMS reader MF). The resulting production of NO_2^- was compared with a known set of $NaNO_2$ standards. Data were normalized with respect to NO_2^- released in the OGD group at time 0 min of re-oxygenation period in each experiment.

Western blot analysis

Slices of each group were lysed in 100 µl ice-cold lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris–HCl, pH 7.5, 1 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, and 1 mM Na_3VO_4). Protein (30 µg) from these lysates were resolved by SDS–PAGE and transferred to Immobilon-P membranes (Millipore Corp.). Membranes were incubated with anti-COX2 (1:1000), anti-iNOS (1:1000), anti-phospho-Ser9-GSK-3β (1:1000), anti-GSK-3β (1:1000), anti- phosphor-Thr308-Akt (1:1000), anti-Akt (1:1000), anti-phospho-Ser136-BAD (1:1000) and anti-β–actin (1:1000). Appropriate peroxidase-conjugated secondary antibodies (1:10000) were used to detect proteins by enhanced chemiluminescence.

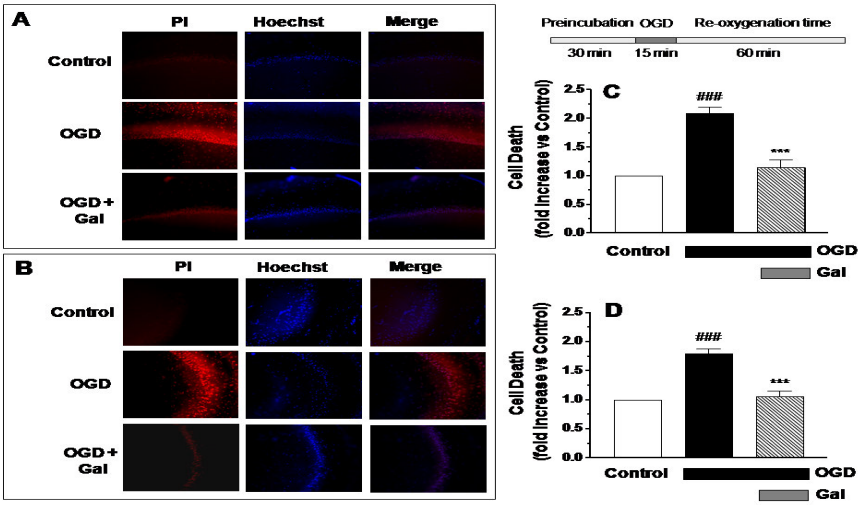

Figure 1. Galantamine reduces cell death in hippocampal slices subjected to OGD and reoxygenation. Slices were subjected to 15 min OGD followed by 1 h re-oxygenation in the absence (OGD) or presence of galantamine 15 µM (Gal); another group of slices were kept during the same period of time with oxygen and glucose (Control). (A) Illustrates fluorescence images of propidium iodide, Hoechst and Merge of CA1. (B) Shows fluorescence images of propidium iodide, Hoechst and Merge of CA3. (C) and (D) Represent cell death measured as the ratio PI/Hoechst and normalized respect to control obtained after the different treatments in CA1 and CA3, respectively. Data are means±s.e.m. of 5 experiments. ###p<0.001 comparing control respect to OGD, ***p<0.001 comparing OGD respect to OGD plus galantamine.

Data Analysis

Data are represented as means ± SEM. Differences between treatments, as a function of drug concentration and re-oxygenation time, were estimated by applying a one way analysis of variance (ANOVA) followed by a Newman-Keuls post-hoc test. Differences were considered to be statistically significant when $p \leq 0.05$. All statistical procedures were carried out using Statistica software version 6.0 for an IBM compatible computer.

Results

Galantamine reduced cell death caused by OGD in CA1 and CA3 hippocampal regions

After subjecting the hippocampal slices to 15 min OGD followed by 60 min re-oxygenation, they were double stained with the fluorescent dies propidium iodide and Hoechst 33342. As shown in **Figures 1A and B,** control slices hardly showed staining for propidium iodide while slices subjected to ischemia markedly increased the number of stained cells. In parallel, a group of hippocampal slices were incubated throughout the experiment with 15 µM galantamine; we selected this concentration because in a previous study from our group we showed that this concentration was the one that afforded maximum protection (Sobrado *et al.*, 2004). Galantamine treatment reduced to almost control levels propidium stained cells (**Figs. 1 A and B**). To evaluate cell death, propidium iodide fluorescence in a given area of CA1 or CA3 was divided by Hoechst fluorescence in the same area; this value was normalized to 1 in control slices. Under these experimental conditions, OGD doubled the amount of cell death in CA1 and CA3 and galantamine treatment reduced to almost control values cell death caused by OGD followed by re-oxygenation. We did not find differences between cell death or protection among areas CA1 and CA3 (**Figs. 1C and 1D**)

Galantamine reduced ROS, NO_2^- production and iNOS induction caused by OGD-reoxygenation

Reactive oxygen species (ROS) are involved in neurotoxicity and neuronal death following an ischemic insult (Coyle *et al.*, 1993). Thus, in this study we examined the effect of galantamine on ROS production, NO release and induction of iNOS in rat hippocampal slices subjected to OGD and re-oxygenation.

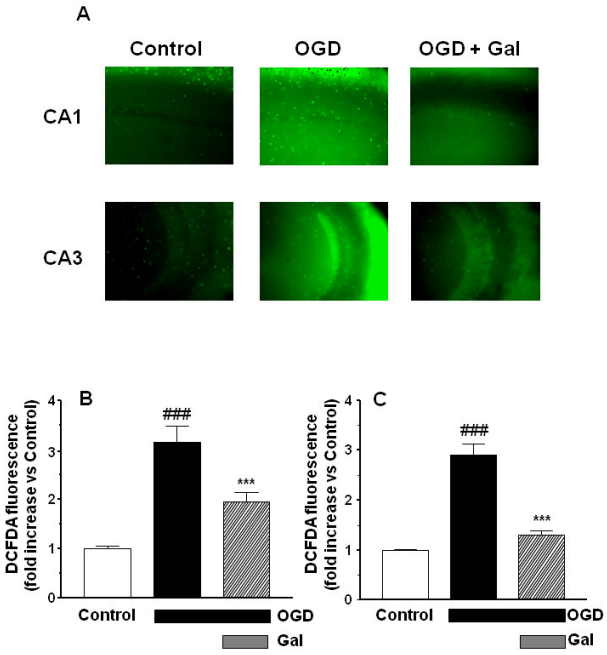

Figure 2. Galantamine reduces ROS production induced by OGD-reoxygenation. Rat hippocampal slices were subjected to 15 min OGD followed by 1 h re-oxygenation in the absence (OGD) or presence of galantamine 15 µM (Gal); another group of slices were kept during the same period of time with oxygen and glucose (Control). ROS production was evaluated in CA1 and CA3 regions as fluorescence of DCFDA. Data were normalized with respect to control that was considered as 1 and represent the mean±s.e.m. of 4 experiments. ###p<0.001 comparing control respect to OGD, ***p<0.001 comparing OGD respect to OGD plus galantamine.

As illustrated in **figure 2**, galantamine markedly reduced ROS production measured in hippocampal slices stained with the fluorescent dye DCFH. OGD followed by reoxygenation increased by 3.15 and by 2.89 fold ROS production in CA1 and CA3, respectively. In galantamine treated slices, ROS production was significantly reduced in CA1 (**Fig 2B**) and CA3 (**Fig 2C**).

We also measured the release of nitrates (NO_2^-) as an indirect measurement of NO production. We followed the protocol shown on top of **Fig. 3A**. Each experiment included slices to measure the basal and the OGD plus re-oxygenation-induced NO_2^- release; parallel slices were exposed to galantamine, which was present during the 30 min of the equilibration period as well as throughout the OGD and re-oxygenation periods. Galantamine reduced NO_2^- release approximately by 53, 54 and 58% at 60, 120 and 180 min of re-oxygenation, respectively (**Fig. 3A**). The reduction was at the limit of statistical significance ($p = 0.05$) just after 60 min OGD but at 60 min 120 and 180 min reoxygenation, reduction was significantly reduced ($p < 0.05$).

In order to understand how NO production could be reduced by galantamine we studied by western blot analysis iNOS production in ischemic slices treated or untreated with galantamine. In **figure 3B**, using the same protocol as in **Fig. 3A**, 60 min OGD followed by 3 h reoxygenation, iNOS increased over two fold with respect to control slices; this increase was reduced to almost basal levels in galantamine treated slices. Moreover, when we used the protocol of 15 min of OGD followed by 60 min re-oxygenation (see protocol on top of **Fig. 1C**), similar results were obtained (**Fig 3C**).

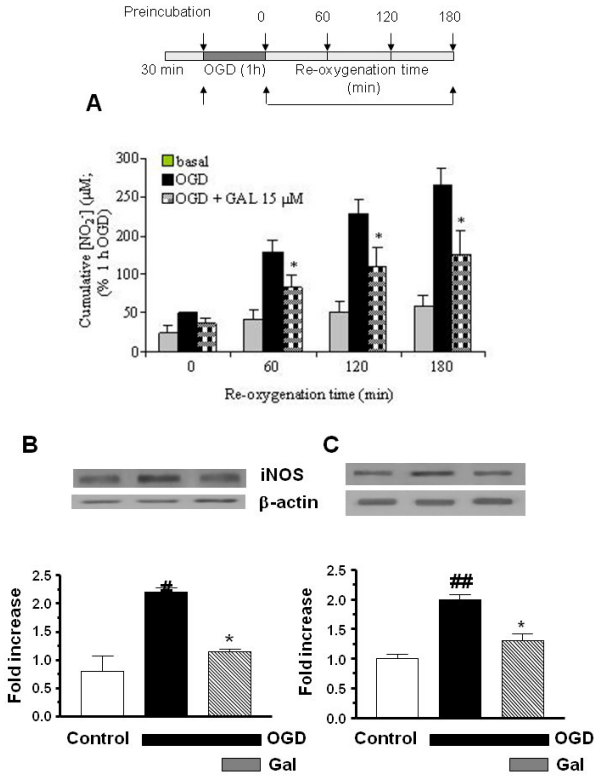

Figure 3. Galantamine reduced NO_2^- production and induction of iNOS. (A) Effects of galantamine (GAL) 15 µM on NO_2^- release from hippocampal slices subjected to 1 h OGD and 3 h re-oxygenation (protocol shown on top). Slices were run in parallel to measure NO_2^- released in basal or OGD conditions, in the absence or the presence of drug. The drug was present during the OGD and the re-oxygenation period. Cumulative release of NO_2^- throughout the experiment is represented as % of NO_2^- released after 1h OGD in control slices. Data are means ± s.e.m. of 6 experiments. *p<0.05 compared with OGD. iNOS expression was determined by western blot analysis, using the same conditions as in A (B), or using the protocol of 15 min OGD followed by 1 h reoxygenation (C). Data correspond to the mean of 3 experiments; all variables were run in parallel. #p<0.05 and ##p<0.01 comparing control respect to OGD, *p<0.05 comparing OGD respect to OGD plus galantamine.

Participation of the survival PI3K-Akt pathway and nicotinic receptors in the protective effects of galantamine

To evaluate the participation of the PI3K/Akt pathway and nicotinic receptors in the neuroprotective mechanism of galantamine we performed OGD-reoxygenation experiments in hippocampal slices treated with galantamine alone or in the presence of LY 294002 or mecamylamine; basal and control OGD-reoxygenation slices were run in parallel. In these slices we first evaluated cell death as the ratio of PI/Hoechst stained cells in CA1 and CA3. At the end of the experiment, the slices were lysed and proteins were extracted to perform western blot analysis for p-Akt and another two proteins that are phosphorylated by Akt i.e. pSer9-GSK-3β and p-Bad.

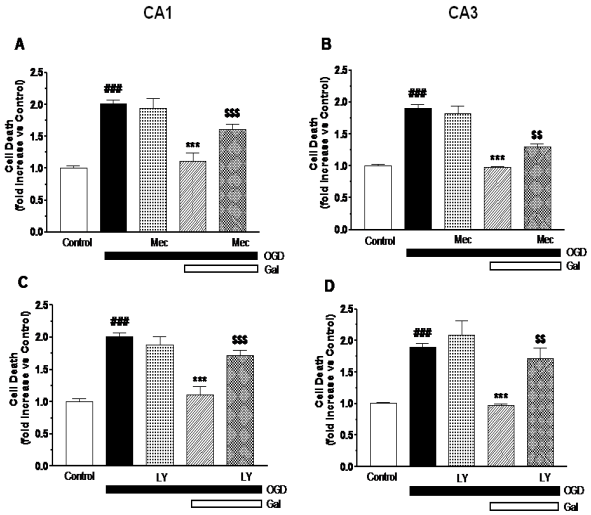

Figure 4. Implication of nicotinic receptors and PI3K/Akt in the protective effect of galantamine. Slices were subjected to 15 min OGD followed by 1 h re-oxygenation. Under these experimental conditions, galantamine 15 µM, alone or in the presence of 30 µM mecamylamine (A and B) or 30 µM LY294002 (C and D) were added to the bath solution 30 min before the OGD period. Cell death was evaluated by quantification of the ratio PI/Hoechst (see Material and Methods) in CA1 and CA3. Data correspond to the mean±s.e.m. of 5 experiments; all variables were run in parallel. ###p<0.001 respect to control, ***p<0.001 OGD-reoxygenation and $$p<0.01, $$$p<0.001 with respect to galantamine.

As illustrated in **figure 4**, the protective effects afforded by galantamine were partially, but significantly, reverted by the nicotinic antagonist (mecamylamine) (**Fig 4A and 4B)** and by the PI3K/Akt inhibitor (LY 294002) in CA1 and CA3 areas (**Fig 4C and 4D**). Neither mecamylamine nor LY294002 showed any significant effect compared to OGD-treated slices. These results indicated that galantamine was indeed acting on nAChRs as well as the intracellular signaling pathway PI3K/Akt to afford protection of the hippocampal neurons subjected to an ischemia-reperfusion stimulus.

To further analyse the PI3K/Akt pathway, at the end of the experiment, we measured p-Akt in the slices subjected to the different experimental conditions evaluated in figure 5. p-Akt was reduced after OGD-reoxygenation and galantamine maintained this values to control values, LY29400 and mecamylamine significantly reduced p-Akt with respect to galantamine treated slices (**Fig. 5A**).

GSK-3β can be phosphorylated in its serine-9 by Akt and is consequently inhibited; inhibition of this enzyme has been reported to afford protection in several models including ischemia. Because of this, we decided to look at pSer9-GSK-3β. As shown in **figure 5B**, pSer9-GSK-3β was significantly reduced after OGD-reoxygenation and galantamine increased to basal levels pSer9-GSK-3β, known to be neuroprotective. LY29400 and mecamylamine prevented galantamine-induced inhibition of GSK-3β.

The pro-apoptotic protein Bad is also phosphorylated and, thereby, inactivated by Akt. In our experimental conditions, OGD-reoxygenation decreased p-Bad levels and galantamine restored them to basal levels. Once again, LY29400 and mecamylamine significantly reduced p-Bad with respect to galantamine (**Fig. 5C**).

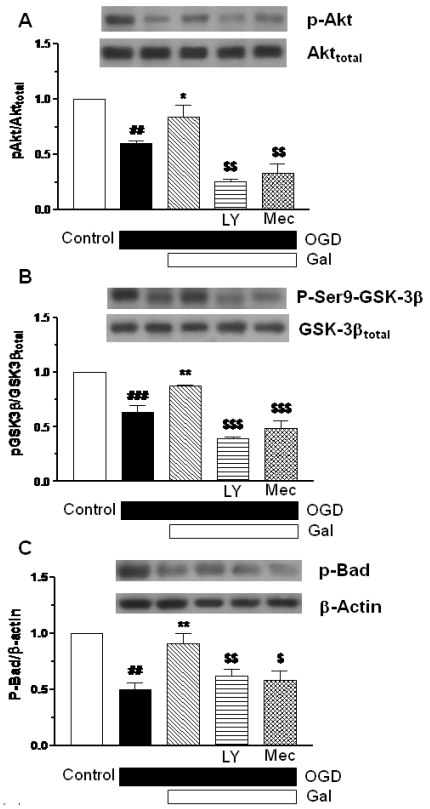

Figure 5. Phosphorylation of Akt, GSK-3b-Ser-9 and Bad. At the end of the experiment represented in figure 5, the slices were taken to measure p- Akt, p-Ser9-GSK-3β and p-Bad by western blot. Data correspond to the mean and s.e.m. of 3 experiments; all variables were run in parallel. ##p<0.01 and ###p<0.001 respect to control; *p<0.05 and **p<0.01 respect to OGD-reoxygenation and $p<0.05, $$p<0.01 and $$$p<0.001 with respect to galantamine.

Discussion and conclusion

In this study we have shown that galantamine can offer a multifunctional mechanism to protect against brain ischemia. The mechanisms related to neuroprotection caused by galantamine in rat hippocampal slices subjected to OGD followed by re-oxygenation implicated: (i) reduction of oxidative stress markers (ROS production, iNOS and NO_2^-) (iii) activation of the survival pathway PI3K/AKt, and consequently, (iii) inhibition of GSK-3β and inactivation of the pro-apoptotic protein Bad.

In this study we have observed almost full protection of hippocampal slices measured as the fluorescence ratio of propidium iodide/Hoechst in CA1 and CA3 hippocampal regions. This result contrasts with the 50% protection observed previously (Sobrado et al., 2004), this difference could be attributed to the protocol used, in the present study we have induced OGD for 15 min followed by 60 min reoxygenation, however, in the previous study OGD was induced for 60 min followed by 3 h re-oxygenation. Most probably the damage caused with 60 min OGD is more pronounced than with 15 min OGD and, consequently, induction of cell defensive mechanisms will be less efficient. The other difference between both studies is the methodology used to evaluate cell death; in this study we have used the fluorescence ratio of propidium iodide/Hoechst which seems to be more sensitive than the measurement of LDH released to the extracellular medium because with 15 min OGD we were hardly able to detect levels of LDH (data not shown). On the contrary, 60 min OGD followed by 3 h re-oxygenation gave such massive cell death, that fluorescence of Hoechst and propidium iodide was difficult to detect.

The idea that galantamine as pre-treatment can be protective against brain ischemic episodes is interesting from a clinical point of view because Alzheimer´s disease patients are being treated with this drug and there are studies that have proven that the occurrence of cerebrovascular disease in these patients can increase progression of dementia (Sheng et al., 2007). On the other hand, poststroke dementia has also been described (Altieri et al., 2004; Barba et al., 2000), therefore galantamine treatment could benefit these patients.

When NO is produced in excessive amount, as occurs during ischemia, it changes from a physiological neuromodulator into a neurotoxic factor. NO release by the novo expression of iNOS contributes to the damage found in ischemia. iNOS knock out mice present smaller infarcts (-30%) and better neurological outcomes after middle cerebral artery oclusion in comparison to their littermates (Iadecola *et al.*, 1995). The mechanism by which iNOS is induced after cerebral ischemia has been related to inflammatory cytokines, oxidative stress and glutamate.

Activation of cyclooxygenase (COX) after brain ischemia-reperfusion contributes to subsequent damage via inflammation and generation of ROS (Cheung et al., 2002). COX catalyses the first two steps in the biosynthesis of prostaglandins and other eicosanoids from arachidonic acid, The inducible COX-2 isoform is constitutively expressed in the brain and kidney and is implicated in inflammation and cytotoxicity (Dubois et al., 1998; Zhang et al., 2002). Under our experimental conditions we were not able to detect significant changes in COX-2 production (data not shown); most probably this was due to the fact that COX-2 is expressed at longer time intervals than the ones used in this study (Nakayama et al., 1998).

Oxidative stress produced during ischemia might itself trigger the induction of iNOS. In fact, a specific pathway for the induction of iNOS under anoxic conditions has been described, and an hypoxic inducible factor-1 (HIF-1) has been characterized in the promoter region of iNOS (Melillo et al., 1995). iNOS is expressed in neurons and in other CNS cells after OGD in rat forebrain slices; this expression occurred at short intervals suggesting that NO can play an important role in tissue damage after brain ischemia (Moro et al., 1998). In the present study we have shown that iNOS can be induced by over two-fold after 1 h OGD followed by 3 h re-oxygenation. Under these experimental conditions, galantamine treatment prevented iNOS induction and this effect related to a marked reduction of ROS production measured as a reduction of DCFH fluorescence in the hippocampal regions CA1 and CA3 and as a reduction in NO_2^- release.

iNOS can also be expressed several hours after the onset of ischemia; therefore, the therapeutic window for administration of iNOS inhibitors has shown to be longer than for nNOS inhibitors. In fact, iNOS inhibitors have been administered starting 12-24 h after middle cerebral artery occlusion, at the time when iNOS was already present, and they reduced infarct volume by 30-40% (Iadecola *et al.*, 1995). The neuroprotective effect induced by iNOS inhibitors against brain ischemia was related to neurological improvement (Nakayama *et al.*, 1998). The latter results are in line with those found in our previous study where we showed that galantamine treatment starting three hours post ischemia protected pyramidal neurons of CA1 of gerbils subjected to global cerebral ischemia (10 min) followed by three days reperfusion; this protective effect also correlated with improved neurological scores (Lorrio et al., 2007).

Activation of NMDA receptors by glutamate, released after an ischemic insult, has also been implicated in the expression of iNOS in rat forebrain slices via a Ca^{2+}-dependent activation of the transcription factor NFkB (Cardenas et al., 2000). In the study by Takada-Takori and co-workers (Takada-Takatori et al., 2006), it is shown that galantamine affords neuroprotection of rat cortical neurons against glutamate neurotoxicity. Therefore, this could be an additional mechanism that could explain the reduction of iNOS in hippocampal slices subjected to OGD in the presence of galantamine.

Signaling through acetylcholine neuronal nicotinic receptors (nAChRs) is being increasingly recognized as playing an important role in different processes such as neurite outgrowth, synaptic transmission, control and synthesis of neurotrophic factors, and neuroprotection (Belluardo *et al.*, 2000; Belluardo *et al.*, 2005; Hernandez *et al.*, 2005). The implication of nAChRs in the protective effects afforded by galantamine have been previously described “in vitro” (Arias et al.,

2004; Arias et al., 2005) and "in vivo" (Lorrio et al., 2007). Activation of the survival PI3K/Akt pathway has been related to nAChRs (Kihara *et al.*, 2001; Shimohama *et al.*, 2001) and also to galantamine´s protective effects (Arias et al., 2005; Kihara et al., 2004). However, these previous studies showed the implication of this signaling pathway in the protective effects of nicotinic receptor activation by nicotine or galantamine against beta-amyloid induced toxicity. In this study we have focused on ischemia-reperfusion neuronal cell death. Under these circumstances, p-Akt was significantly reduced and the protective drug, galantamine, maintained p-Akt to basal levels in the hippocampal slices. These effects were related to PI3K/Akt and to nicotinic receptors because both LY294002 and mecamylamine blocked the actions of galantamine, both on cell survival and p-AKt.

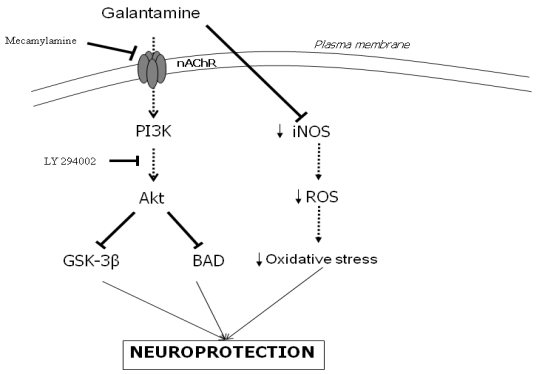

Figure 6. Schematic diagram illustrating the putative points of regulation by galantamine during hippocampal oxygen and glucose deprivation (OGD) to cause neuroprotection.

To further analyze the downstream signaling pathway activated by galantamine to afford protection against OGD-reoxygenation, we focused on GSK-3β and Bad because both are substrates of Akt. GSK-3β phosphorylated at the tyrosine 216 (Tyr^{216}) residue is increased following middle cerebral artery occlusion indicating the presence of the active enzyme (Bhat et al., 2004). However, phosphorylation of its residue Ser-9, inactivates it. GSK-3β inhibition has been shown to be protective against a plethora of neurological insults including cerebral ischemia (Bhat *et al.*, 2004; Kamada *et al.*, 2007; Kelly *et al.*, 2004; Manji *et al.*, 1999) suggesting that an inhibition of GSK-3β improves brain cell survival. OGD-reoxygenation reduced p-Ser9-GSK-3β and galantamine prevented its inhibition; this effect was related to PI3K/Akt and nicotinic receptors because both LY294002 and mecamylamine reduced galantamine´s protection and GSK-3β inhibition. A parallel pattern was observed for p-Bad, a proapoptotic protein that inactivates when phosphorylated by Akt (del Peso et al., 1997).

Inactivation of Bad, besides the already described induction of the antiapoptotic protein Bcl-2 (Arias et al., 2004) and prevention of Caspase-3 cleavage (Lorrio et al., 2007), could further account for the anti-apoptotic actions described for galantamine. The protection induced by galantamine was not completely reverted by LY294002 and mecamylamine, indicating that, most probably, several mechanisms are implicated in its protective effect against ischemia.

In conclusion, galantamine can afford neuroprotection of hippocampal slices against OGD-reoxygenation by a dual mechanism that includes: (i) reduction of oxidative stress by reducing iNOS and ROS production and, (ii) by activating the survival PI3K/Akt pathway, that in turn inhibits GSK-3β and Bad, by phosphorylation (Fig. 6).

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NEUROPROTECTION AFFORDED BY NICOTINE AGAINST OXYGEN AND GLUCOSE DEPRIVATION IN HIPPOCAMPAL SLICES IS LOST IN α7 NICOTINIC RECEPTOR KNOCKOUT MICE

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Abstract—Although α7-receptors are considered the main target for neuroprotection, other receptor subtypes (α4β2 or α3β4) have also been implicated. Hence, we have used α7-transgenic mice, to study the hypothesis that α7-receptors play a dominant role in mediating neuroprotection in an *in vitro* model of ischemia. We have used rat and mouse hippocampal slices to establish the model of nicotinic neuroprotection against oxygen and glucose deprivation (OGD). Neuronal damage caused by OGD during 1 h plus 3 h re-oxygenation, was quantified by measuring lactate dehydrogenase (LDH) release from hippocampal slices. In rat hippocampal slices, OGD increased over twofold basal LDH release. Such increase was reduced when treated with 10–100 μM nicotine; maximal protection afforded by nicotine amounted to 46%. This neuroprotection was antagonized by the non-selective nicotinic receptor for acetylcholine (nAChR) blocker mecamylamine (10 μM). In hippocampal slices from wild-type control mice, nicotine (100 μM) decreased by 54.4% LDH release evoked by OGD plus re-oxygenation. In contrast, nicotine failed to exert neuroprotection in α7 knockout mice. This finding reinforces the view that the hippocampal neuroprotective effects of nicotine are predominantly linked to α7 receptors.

Key words: nicotine, oxygen-glucose deprivation, hippocampal slices, α7-knockout mice, neuroprotection.

Neuronal nicotinic receptors for acetylcholine (nAChRs) are highly concentrated in the hippocampus, thalamus, and cerebral cortex (Clarke et al., 1985; Wada et al., 1989; Hogg et al., 2003). A great deal of interest has evolved around the hypothesis that several nAChR subtypes might be involved in the neuroprotective mechanisms against neuronal damage produced by various noxious stimuli. The clearest data in this direction emerge from studies performed in neuronal cultures, where nicotinic agonists exhibit neuroprotective effects against glutamate (Donnelly-Roberts et al., 1996; Stevens et al., 2003; Gahring et al., 2003; Sun et al., 2004), trophic factor deprivation (Yamashita and Nakamura, 1996), β-amyloid (Gahring et al., 2003; Kihara et al., 1997; Liu and Zhao, 2004; Moore et al., 2004), or hypoxia (Hejmadi et al., 2003).

More dubious results emerge from *in vivo* models of cerebral ischemia. For instance, the α7 nAChR selective agonist GTS-21 (Kem, 2000) protects against neuronal death elicited by cerebral ischemia in gerbils but surprisingly, nicotine affords no neuroprotection; this contrasts with the fact that both nicotine and GTS-21 mitigate the memory deficits induced by such ischemic insult (Nanri et al., 1998). On the other hand, nicotine provides protection against CA1 hippocampal neuronal death after transient cerebral ischemia in the rat (Kagitani et al., 2000). Even fewer studies are yet available in hippocampal slices subjected to oxygen and glucose deprivation (OGD) followed by a re-oxygenation period. To our knowledge, the only study where the topic of nicotinic receptor involvement in neuroprotection was approached was with galantamine (Sobrado et al., 2004), an allosteric potentiating ligand (APL) that augments the effects of acetylcholine (ACh) on nicotinic receptors (Maelicke and Albuquerque, 2000).

The question of which nAChR subtype(s) is involved in the neuroprotective effects of nicotinic agonists is highly relevant in the context of the design and development of selective nicotinic receptor ligands that might eventually reach the clinic for the treatment of stroke. As far as we know, attempts to answer this question have been carried out only in neuronal cultures. For instance, the non-selective blocker of nAChRs mecamylamine and the β2 selective blocker dihydro-β-erythroidine, reversed the neurotoxic effects of hypoxia, suggesting that α4β2 as well as α7 and other receptor subtypes, could be involved in the neuroprotective effects of nicotinic agonists (Hejmadi et al., 2003; Kaneko et al., 1997). Also, two selective α7 receptor blockers, methyllycaconitine (MLA) and α-bungarotoxin mitigate the neuroprotective effects of nicotine against glutamate neurotoxicity (Donnelly-Roberts et al., 1996; Kaneko et al., 1997) and hypoxia (Hejmadi et al., 2003); this suggests that α7 nAChRs mediate such neuroprotective effects.

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Abbreviations: LDH, lactate dehydrogenase; mOD, mean optical density; nAChR, nicotinic receptor for acetylcholine; OGD, oxygen and glucose deprivation; +/+, wild type; −/−, nAChR knockout.

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In bovine chromaffin cells, that among others, express α3β4 and α7 receptors; non-selective and α7-selective antagonists reversed the neuroprotective effects of galantamine (Arias et al., 2005).

Although various subtypes of nAChRs have been involved in neuroprotective mechanisms, the α7 subtype is emerging as a clear target for the development of neuroprotective drugs. This is why we decided to test this hypothesis taking advantage of having in our laboratory viable knockout mice for the nAChR α7 gene (Orr-Urtreger et al., 1997). Because the scarcity of α7 knockout mice (they reproduce very slowly), we first decided to prove that nicotine had neuroprotective effects in rat hippocampal slices subjected to OGD, and that those effects were reversed by mecamylamine. Once validated, this model was extrapolated to α7 wild-type and knockout mice; the neuroprotective effects of nicotine observed in control mice were lost in mice with ablation of the α7 gene, indicating that the α7 nAChR is indeed participating in the neuroprotective effects of nicotine in an *in vitro* model of brain ischemia.

EXPERIMENTAL PROCEDURES

Some experiments were performed using the brain of adult male Sprague–Dawley rats (275–325 g) from a colony of our animal quarters. Other experiments were conducted on 18-month-old α7 nAChR mutant mice. The mice were descendants of mice constructed by Orr-Urtreger et al. (1997). The α7 null mutant was derived at Baylor College of Medicine in 129ISvEv ES cells and the mutated gene was backcrossed onto the C57BII6J mouse strain. The progeny for the testing was generated by matings of α7 +/ mice, and both females and males were included in our experiments. All animal assays were carried out following the European Community Council Directive issued for these purposes and were approved by the Ethics Committee of the Facultad de Medicina, Universidad Autónoma de Madrid. All efforts were made to minimize the number of animals used and their suffering.

Animals were deeply anesthetized by i.p. injection of sodium pentobarbital (60 mglkg), decapitated, and each brain was rapidly removed from the skull and placed into ice-cold Krebs-bicarbonate dissection buffer (pH 7.4) containing, in mM: NaCl 120, KCl 2, $CaCl_2$, 0.5, $NaHCO_3$ 26, $MgSO_4$ 10, KH_2PO_4 1.18, glucose 11, and sucrose 200 (Moro et al., 2000). All chamber solutions were pre-bubbled with either 95% O_2/5% CO_2 or 95% N_2/5% CO_2 gas mixtures, for at least 45 min before slice immersion, to ensure O_2 saturation, or O_2 removal, and the pH of the solutions. The hippocampi were quickly dissected, glued down leaning vertically against agar blocks in a small chamber, submerged in cold oxygenated dissection buffer and sectioned in transverse slices of 350 μm thick for neuroprotection studies (dissection period). Hippocampi were cut into slices using a vibratome (TS1000 Leica; Heidelberg, Germany). Cutting parameters used were 0.5 mm/s, 60 Hz and 0.8 mm amplitude. Immediately after vibratome sectioning, the slices (the two first most anterior slices were discarded) were transferred to a vial of sucrose-free dissection buffer, bubbled with 95% O_2l5% CO_2 at room temperature for 60 min to recover from slicing trauma, before starting the experiments (equilibration period). The slices were completely submerged and protected from the vigorous bubbling in the chamber by a semipermeable nylon mesh.

After an initial preincubation period of 30 min, the slices corresponding to the basal group were incubated 60 min in a normal Krebs solution, having the following composition in mM: NaCl 120, KCl 2, $CaCl_2$ 2, $NaHCO_3$ 26, $MgSO_4$ 1.19, KH_2PO_4 1.18 and glucose 11; this solution was equilibrated with 95% O_2/5% CO_2. OGD was induced by incubating the slices for a 60 min period in a glucose-free Krebs solution, equilibrated with a 95% N_2l5% CO_2 gas mixture; glucose was replaced with 2-deoxyglucose (Sigma-Aldrich, Madrid, Spain). After this OGD period, the slices were returned to an oxygenated normal Krebs solution containing glucose (re-oxygenation period). These experiments were performed at 37 °C. When used, (–)-nicotine hydrogen tartrate (100 μM) and mecamylamine (10 μM) were added to the OGD slices during the 30 min of preincubation period and remained there during the OGD and re-oxygenation period. Nicotine and mecamylamine were purchased from Sigma. Drugs were all dissolved in water at high concentrations (nicotine at 100 mM and mecamylamine at 10 mM) and added to buffers immediately before the experiments.

The viability of hippocampal slices after a given treatment was monitored by measuring the activity of the lactate dehydrogenase (LDH) released into the incubation media. This is a technique that has been proven to be reliable as a measure of neuronal cell death in rat hippocampal slices (Sobrado et al., 2004; Koh and Choi, 1987). Samples of this solution were taken after OGD and at times 0 (OGD) and 1, 2, and 3 h of the re-oxygenation period (see protocol in Fig. 1A). LDH activity was measured spectrophotometrically at 490–620 nm, using a microplate reader (Labsystems iEMS reader MF). The remaining LDH was obtained by incubating the slices with 1% Triton X-100 at the end of the experiment, for 30 min; then, the samples were centrifuged and an aliquot from the supernatant was taken to measure the intracellular LDH. LDH levels remaining in the basal and OGD-slices after 3 h of reperfusion were high and not significantly different from each other (Sobrado et al., 2004). Thus, only the LDH released into the medium was measured.

Data are represented as means ± S.E.M. Differences between treatments, as a function of drug concentration and re-oxygenation time, were estimated by applying a one-way ANOVA followed by Duncan's test. Differences were considered to be statistically significant when $P<0.05$.

RESULTS

LDH release as an index of neuronal damage in rat hippocampal slices subjected to OGD and re-oxygenation

Rat hippocampal slices were sequentially incubated with various solutions following the protocol shown in Fig. 1A. Fig. 1B shows the amount of LDH released into the bathing fluid in basal slices that were constantly oxygenated (white columns) and in slices that suffered 1-h period of OGD; during the 0.5-h pre-incubation period, LDH release was similar in basal and OGD slices. LDH release significantly augmented above basal during the 1-h OGD period; the enzyme release gradually diminished during each of the second and third subsequent re-oxygenation 1-h periods. In Fig. 1C, LDH release has been plotted in a cumulative manner, adding the amount of enzyme found in a given period to the next starting from the OGD period, and so forth.

Nicotine mitigates the OGD-elicited neuronal damage in rat hippocampal slices, by acting on nicotinic receptors

Each experiment in this series was performed in five groups of hippocampal slices from the same rat that were run in parallel. The first group of slices served to

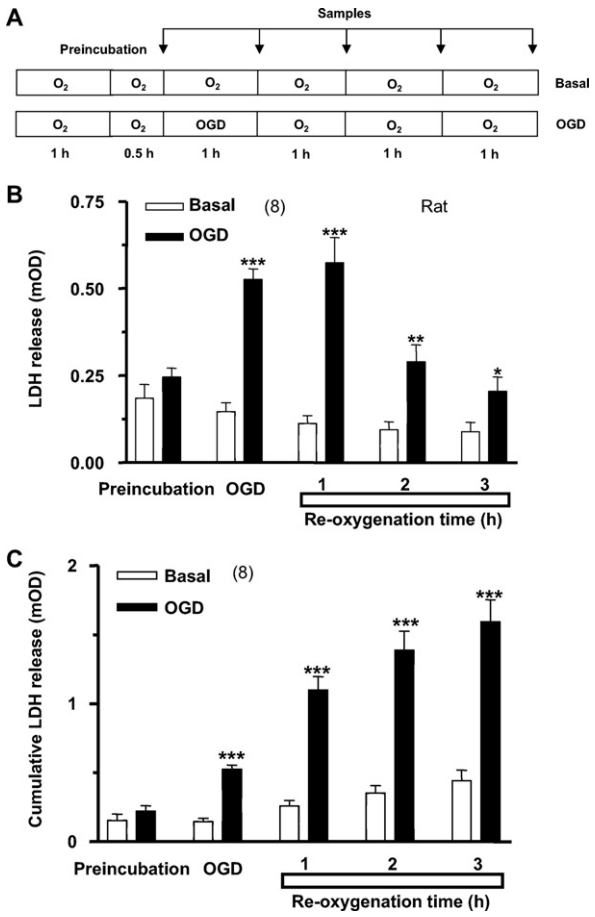

Fig. 1. Release of LDH into the medium as an index of neuronal damage caused by OGD. In each experiment, two groups of rat hippocampal slices were run in parallel following the protocol shown in panel A. One group was maintained along the entire experiment in Krebs-bicarbonate solution equilibrated with 95% O_2/5% CO_2 at 37 °C (basal LDH release); the other was subjected to a 1-h OGD period, followed by re-oxygenation (three 1-h periods in oxygenated solution) (OGD-evoked LDH release). Before OGD, the slices were incubated during 1 h in oxygenated Krebs-bicarbonate solution (stabilization period) to allow their equilibration to the experimental conditions, and during 0.5 h (pre-incubation period). The ordinate in panel B shows the LDH in mOD units recovered in the incubation media collected in each period (arrows in panel A). Panel C shows the cumulative values of LDH released (in ordinate) at various incubation periods both, in basal and ODG periods. Data are means S.E.M. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ as compared with basal slices in their respective time period ($n=8$).

estimate the basal LDH release and the second to monitor the OGD-induced increase of LDH release, according to the protocol shown in Fig. 1A. The other three groups were pre-incubated 30 min with nicotine (10, 30, or 100 μM); nicotine was also present along the OGD and re-oxygenation periods (see protocol in Fig. 2A).

Fig. 2B shows that LDH activity in the media of the 0.5 h pre-incubation period was similar for all five groups, around 0.2 mean optical density (mOD) units. During OGD, LDH release increased 3.6-fold above basal. The three concentrations of nicotine decreased the amount of LDH released in the OGD slices ($P<0.05$ for nicotine 10 and 30 μM; $P<0.01$ for nicotine 100 μM).

During re-oxygenation, LDH accumulated gradually in the media. However, such accumulation was about 30–40% lower in the preparations bathed with 10–100 μM nicotine ($P<0.01$).

Fig. 2C shows pooled results from experiments that were individually performed by running in parallel five groups of hippocampal slices from the same rat. Once more, groups of slices 1 and 2 were used to estimate basal and OGD-elicited LDH release. A third group of slices was incubated with 100 μM nicotine, the fourth with 10 μM mecamylamine (a non-selective nAChR blocker) (Kihara et al., 1997), and the fifth with 100 μM nicotine plus 10 μM mecamylamine. As above, drugs were present 30 min prior to

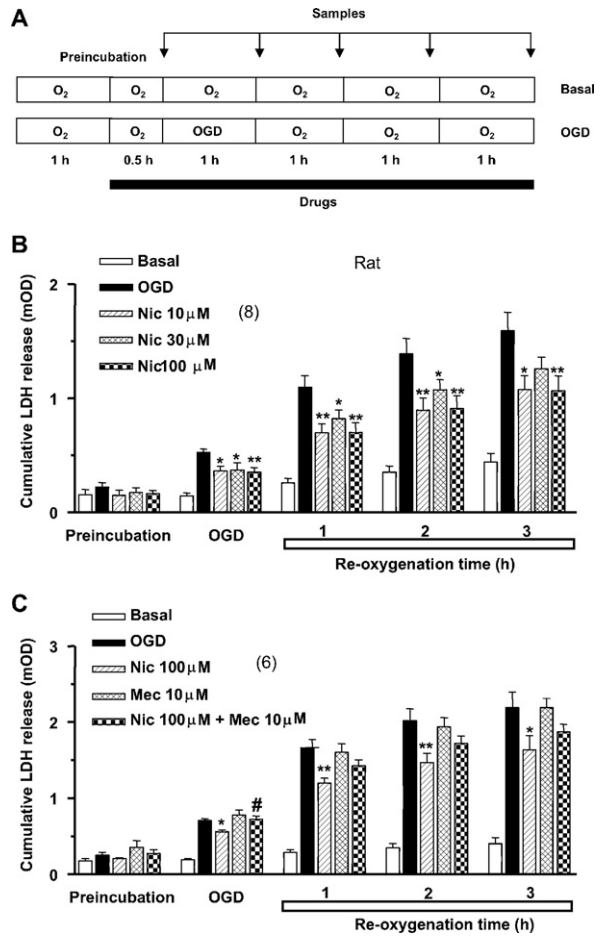

Fig. 2. Nicotine (Nic) reduces the amount of LDH released from rat hippocampal slices subjected to OGD, and mecamylamine (Mec) antagonizes such an effect. The experiments were done according to the protocol shown in panel A; drugs were added as shown by the horizontal black bar and samples of incubation media were collected during the periods indicated by the arrows on top. Each experiment of panels B and C was performed with hippocampal slices taken from the same rat. One group of slices was used to explore the LDH released along the experiment in basal conditions, and the other to study the effects of OGD in the absence (OGD) or the presence of Nic at the indicated concentrations. In panel C the five groups of slices were incubated in the absence or the presence of 100 μM Nic, 10 μM Mec or Nic plus Mec. Data are expressed as cumulative LDH release, as explained in Fig. 1C legend (ordinates); they are means S.E.M. * $P<0.05$, ** $P<0.01$ compared with OGD ($n=8$) and # $P<0.05$ as compared with Nic-treated slices ($n=6$).

OGD, during OGD, and during the 3-h re-oxygenation period. Note that nicotine alone significantly mitigated the increase of LDH caused by OGD, during the OGD as well as during the three 1-h re-oxygenation periods. Mecamylamine reversed the neuroprotective effects of nicotine by 40%. This suggests that in rat hippocampal slices, nicotine is indeed offering neuroprotection against OGD damage and that such effect is mediated by nicotinic receptors.

LDH release evoked by OGD in hippocampal slices from wild-type (α7 +/+) and α7 nAChR knockout mice (α7 -/-)

First we validated in the mouse tissue, the protocol used in the rat; we studied LDH release as an indication of neuronal damage caused by OGD. As far as we know, this information is unavailable in mouse hippocampal slices.

Slices from α7 +/+ and α7 −/− mice were subjected to a sequential series of incubation periods according to the protocol shown in Fig. 1A. Note the low level of LDH released during the pre-incubation period in both α7 +/+ (Fig. 3A) and α7 −/− mice (Fig. 3B). OGD significantly enhanced LDH release above basal (about 10-fold), in α7 +/+ mice; the enzyme was further enhanced above basal by 15-fold, sixfold, and fourfold at 1, 2, and 3 h re-oxygenation, respectively (Fig. 3A). A similar pattern of LDH release elevations in OGD-treated slices was seen in α7 −/− mice (Fig. 3B). Thus, the enzyme rose ninefold above basal during the OGD period; during re-oxygenation, the enzyme rose 10-fold, sixfold, and threefold above basal, respectively after 1, 2, and 3-h re-oxygenation. Hence, the measurement of LDH release to the extracellular medium, that has proven to be a reliable index of neuronal damage in rat hippocampal slices in two previous papers (Koh and Choi, 1987; Sobrado et al., 2004) and in this study, is also a valid index of cell death in mouse hippocampal slices.

Nicotine affords neuroprotection against OGD-induced neuronal damage in α7 +/+ mice but not in α7 -/- mice

Once the neurotoxicity technique was validated in the mice, we used this model to test whether the neuroprotective effects of 100 μM nicotine seen in rats were also maintained in mice hippocampal slices.

Each individual experiment was done in parallel with hippocampal slices of a α7 +/+ mouse and a α7 −/− mouse. Nicotine was present during the last 30 min of the pre-incubation period and along the rest of the experiment. In α7 +/+ slices, cumulative LDH activity remained very low (below 0.1 mOD) along the different periods of the experiment; LDH release, however, augmented significantly above basal during the OGD and the re-oxygenation periods. Note that the slices treated with nicotine released significantly smaller amounts of LDH (Fig. 4A). The reduction was pronounced (around 54%) at time periods including OGD and re-oxygenation ($P<0.001$, compared with OGD).

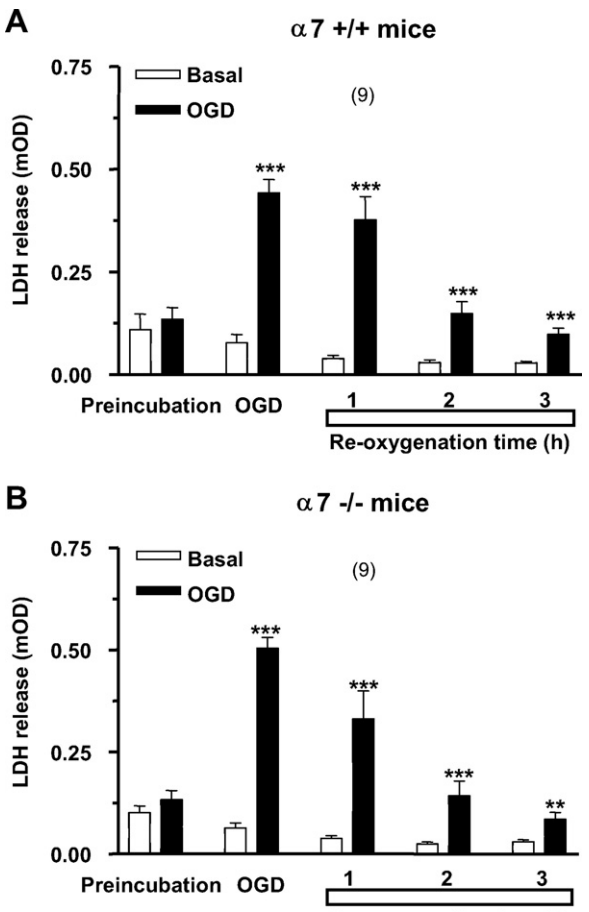

Fig. 3. Augmentation of the LDH released into the media bathing hippocampal slices subjected to OGD, from wild-type mice (α7 +/+) and α7 nAChR-deficient mice (α7 -/-). The experiments were individually run in two parallel groups of slices from the same animal; one was employed to measure basal LDH release and the other to monitor LDH released by OGD and re-oxygenation (see protocol in Fig. 1A). Panel A shows the LDH released in each incubation period, in slices from α7 +/+ mice; panel B shows similar experiments performed with slices from α7-deficient mice. Data are means S.E.M. ** $P<0.01$ and *** $P<0.001$ as compared with basal (n 9).

As in α7 +/+ slices, in α7 −/− slices basal LDH release was quite low and was maintained below 0.1 mOD along the experiment. LDH release increased over ninefold during the OGD period and augmented significantly during the re-oxygenation periods. Interestingly, in α7 −/− slices LDH release induced by OGD was not significantly reduced in nicotine-treated slices (Fig. 4B). Thus, nicotine did not protect in α7 −/− slices subjected to OGD.

DISCUSSION

We have found in this study that nicotine afforded neuroprotection against the neurotoxic effects of OGD in rat hippocampal slices. Neuroprotection was also observed in hippocampal slices of control mice; however, nicotine failed to offer neuroprotection in knockout mice for the α7 nAChR gene. As stated in the introduction, there is

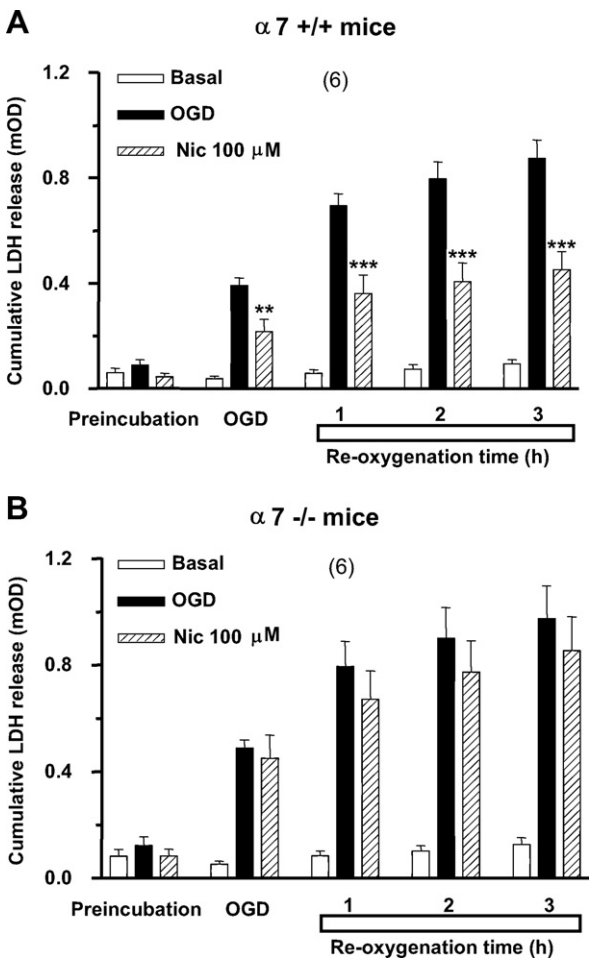

Fig. 4. Nicotine (Nic) provides neuroprotection against OGD damage in hippocampal slices from wild-type, but not in α7 nAChR-deficient mice. The experiment was run according to the protocol of Fig. 2A. Because of the scarcity of hippocampal tissue in mice, only the highest concentration of Nic used in the rat (100 μM), was used here. Three groups of slices from each animal were run in parallel in every individual experiment: one served to explore the basal LDH release, the second to measure the OGD-evoked release in the absence of Nic, and the third to measure LDH release evoked by OGD in the presence of Nic. LDH was cumulatively expressed in mOD units (ordinates), as in Fig. 1C. Data are means ± S.E.M. of six wild-type mice (panel A) and six nAChR-deficient mice (panel B). ** $P<0.01$ and *** $P<0.001$ compared with OGD in the absence of Nic (OGD).

growing evidence suggesting an important role of nAChRs in mediating neuroprotection against various types of neurotoxic stimuli. In this context, the fact that mecamylamine prevented the neuroprotection afforded by nicotine, suggests the involvement of nicotinic receptors in the OGD model of neurotoxicity in hippocampal slices.

Nicotine did not afford statistically significant neuroprotection in α7 −/− mice. It is known that the suppression of the gene for a given receptor subtype or ion channel is often compensated by the over-expression of another receptor subtype of the same class. This is the case for voltage-activated Ca^{2+} channels; thus, in α_{1A} knockout mice the lack of P/Q channels is compensated by an over-expression of L-type Ca^{2+} channels (Aldea et al., 2002). Perhaps the functions of α7 receptors in knockout mice might be overtaken by α4β2 or other subtypes of nAChR. However, this does not seem to be the case for the neuroprotective effects described for the α7 nAChRs because nicotine did not protect in α7 −/− mice. These results indicate that the α7 receptor subtype is predominant in the protective effect of nicotine in hippocampal slices subjected to OGD.

Our data on α7 −/− mice reinforce the prominent role of α7 nAChRs in sustaining neuronal viability against a given neurotoxic stimulus. As stated in the introduction, various subtypes of nAChRs (α7, α4β2, α3β4) have been implicated in neuroprotective mechanisms (see references in the introduction). Those studies have used antagonists for nAChR subtypes i.e. α-bungarotoxin and methyllycaconitine for α7 receptors, dyhydro-β-erythroidine for α4β2 receptors, and mecamylamine for all types of receptors. Although this pharmacological strategy is valuable, we believe that knocking out a specific receptor gene provides more compelling evidence to define the role of such receptor in a given function. Knocking out a receptor subtype gene might give rise to compensation with other closely related gene products and so, the function is not apparently altered, as stated before. However, this is not the case for nicotine-elicited neuroprotection that is fully lost in α7 deficient mice. This constitutes good evidence for α7, but not for other nAChR subtypes, in nicotinic receptor-mediated neuroprotection in the mouse hippocampus.

Our data are relevant in the context of the potential development of selective α7 nAChR agonists to treat stroke. An increasing number of selective non-peptide agonists are available, i.e. GTS-21 (Nanri et al., 1998), JAY 2-21-29 (Buccafusco et al., 2004), and choline (Alkondon et al., 1997; Fuentealba et al., 2004). It will be interesting to see whether those compounds offer protection in hippocampal slices subjected to OGD. Hence, our results with OGD suggest that α7 nAChR agonists might be useful in the acute treatment of stroke.

Mice homozygous for the α7 nAChR null mutation lack detectable mRNA, α-bungarotoxin binding sites, and fast hippocampal nicotinic currents. However, the mice are viable and anatomically normal (Orr-Urtreger et al., 1997). It seems that α7 receptors are not necessary for hippocampal-dependent learning or sensorimotor gating (Paylor et al., 1998) neither for the nicotine-induced seizures (Franceschini et al., 2002) or nicotine withdrawal somatic signs (Grabus et al., 2005). However α7 −/− mice have altered baroreflex responses, indicating a role in the control of autonomic nervous system reflexes (Franceschini et al., 2000). Also, female null mutant mouse have asynchronous estrous cycles and a reduced number of surviving pups (Morley and Rodriguez-Sierra, 2004); this explains why our mouse colony has a low reproduction rate. Furthermore, nicotine withdrawal produced hyperalgesia in α7 −/−, but not in α7 +/+ mice (Grabus et al., 2005). To this grow-

ing data on the physiological roles of α7 receptors, derived from α7 null mice, we add our present data showing a new relevant role for α7 nicotinic receptors, that of neuroprotection of hippocampal neurones.

CONCLUSION

In conclusion, we have shown that nicotine affords protection in rat and mouse hippocampal slices subjected to OGD; the protection was greater in the mouse as compared with rat slices. The protection was linked to nAChR activation. The fact that nicotine protection was significantly lost in α7 nAChR-deficient mice, clearly illustrates that α7 receptors play a dominant role in nAChR agonist-mediated neuroprotection.

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Nicotinic receptor activation by epibatidine induces heme oxygenase-1 and protects chromaffin cells against oxidative stress

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Abstract

Activation of neuronal nicotinic acetylcholine receptors (nAChR) provides neuroprotection against different toxic stimuli that often lead to overproduction of reactive oxygen species (ROS) and cell death. ROS production has been related with disease progression in several neurodegenerative pathologies such as Alzheimer's or Parkinson's diseases. In this context, we investigated here if the exposure of bovine chromaffin cells to the potent nAChR agonist epibatidine protected against rotenone (30 μ mol/L) plus oligomycin (10 μ mol/L) (rot/oligo) toxicity, an in vitro model of mitochondrial ROS production. Epibatidine induced a concentration- and time-dependent protection, which was maximal at 3 μ mol/L after 24 h. Pre-incubation with dantrolene (100 μ mol/L) (a blocker of the ryanodine receptor channel), chelerythrine (1 μ mol/L) (a protein kinase C inhibitor), or PD98059 (50 μ mol/L) (a MEK inhibitor), aborted epibatidine-elicited cytoprotection. Mitochondrial depolarization, ROS, and caspase 3 active produced by rot/oligo were also prevented by epibatidine. Epibatidine doubled the amount of heme oxygenase-1 (HO-1), a critical cell defence enzyme against oxidative stress. Furthermore, the HO-1 inhibitor Sn(IV) protoporphyrin IX dichloride reversed the epibatidine protecting effects and HO-1 inducer Co (III) protoporphyrin IX dichloride exhibited neuroprotective effects by itself. The results of this study point to HO-1 as the cyto-protective target of nAChR activation through the following pathway: endoplasmic reticulum Ca^{2+}-induced Ca^{2+}-release activates the protein kinase C/extracellular regulated kinase/HO-1 axis to mitigate mitochondrial depolarization and ROS production. This study provides a mechanistic insight on how nAChR activation translates into an antioxidant and antiapoptotic signal through up-regulation of HO-1.

Keywords: epibatidine, extracellular regulated kinase 1/2, heme oxygenase-1, neuronal nicotinic acetylcholine receptors, neuroprotection, reactive oxygen species.

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0xidative stress is a leading mechanism of cell death in distinct cytotoxic models such as glutamate (Parfenova et al. 2006), β-amyloid (Tamagno et al. 2006), or hydrogen peroxide-induced cytotoxicity (Kim et al. 2005). It has also been involved in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (AD and PD) (Mariani et al. 2005) and stroke (Saito et al. 2005). 0verproduction of reactive oxygen species (R0S) such as superoxide free radicals or hydrogen peroxide, lead to damage of both

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Abbreviations used: AD, Alzheimer disease; BV, biliverdin; CFDA, 6-carboxyfluorescein diacetate; CICR, Ca^{2+}-induced Ca^{2+} release; CoPP, Co (III) protoporphyrin IX dichloride; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; ERK, extracellular regulated kinase; H_2DCFDA, 2ℓ,7ℓ-dichlorofluorescein diacetate; H0, heme oxygenase; LDH, lactate dehydrogenase; MAP, mitogen-activated protein; nAChR, neuronal nicotinic acetylcholine receptors; PKC, protein kinase C; R0S, reactive oxygen species; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SnPP, Sn(IV) protoporphyrin IX dichloride; TMRE, tetramethylrhodamine ethyl ester.

neuronal and vascular cells by cell membrane lipid destruction and DNA cleavage (Wang et al. 2003).

Heme oxygenase (H0) is the rate-limiting enzyme that degrades the pro-oxidant heme group and produces equimolecular quantities of carbon monoxide (C0), iron, and biliverdin (BV). BV is subsequently reduced to bilirubin by BV reductase. These three by-products have been related to cell protection in distinct cellular models (Kim et al. 2005; Vitali et al. 2005). An inducible isoform H0-1 and a constitutive isozyme H0-2 have been described. H0-1 is induced in response to a great variety of stress-inducing pathological conditions (Keyse and Tyrrell 1987; Nimura et al. 1996). Moreover, studies in H0-1-deficient mice have confirmed that the H0 system is indispensable for cell protection against oxidative stress (Poss and Tonegawa 1997). In neurons, there is a low expression of H0-1 (Maines 2004), and its induction is related with protection against H_2O_2 and hydrogen-peroxide (Kim et al. 2005), focal ischemia (Nimura et al. 1996), and glutamate (Parfenova et al. 2006). Furthermore, in postmortem brains of AD patients, there was H0-1 induction in neurons of the cerebral cortex and the hippocampus, and H0-1 was co-localized with neurofibrillary tangles (Schipper et al. 1995). Therefore, it is generally accepted that H0-1 represents a physiological protective mechanism against oxidative stress.

Nicotinic agonists have been widely tested for neuroprotection in an in vitro and in vivo models against distinct neurotoxic insults, i.e. glutamate (Stevens et al. 2003; Sun et al. 2004), β-amyloid (Gahring et al. 2003; Arias et al. 2005), hypoxia (Hejmadi et al. 2003), oxygen and glucose deprivation (Egea et al. 2007), and focal ischemia (Kagitani et al. 2000). In fact, chronic pre-incubation with nicotine causes protection of neurons against these different noxious stimuli. Usually, these studies have been focused to study the over-expression of anti-apoptotic proteins such as Bcl-2 and the pathways involved in such protection. However, little attention has been paid to how activation of nicotinic receptors can induce antioxidant enzymes when the cell is subjected to an oxidative stress condition.

Because of this, we focused our study on how epibatidine, a potent nicotinic agonist (Badio and Daly 1994), could modify the expression of antioxidant enzymes. We selected as cytotoxic stimulus a model of R0S production, the combination of rotenone plus oligomycin-A (rot/oligo) that inhibit mitochondrial respiration complex I and V, respectively (Hoglinger et al. 2005). We have used bovine adrenal chromaffin cells because they have become an interesting model to study mechanisms of neuronal death and protection (Maroto et al. 1994; Arias et al. 2004) and they express a rich collection of nicotinic receptor subunits i.e. α3, α5, α7, and β4 (Campos-Caro et al. 1997).

We have found that epibatidine afforded cytoprotection through an intracellular biochemical pathway that implies Ca^{2+} entry by neuronal nicotinic acetylcholine receptors (nAChR) activation, Ca^{2+}-induced Ca^{2+} release (CICR) from the endoplasmic reticulum (ER), protein kinase C (PKC) and mitogen-activated protein (MAP) kinase activation, and H0-1 over-expression. To our knowledge, this is the first study involving H0-1 in the neuroprotectant actions of nAChR agonists.

Materials and methods

Materials

Rotenone, oligomycin A, (±) epibatidine dihydrochloride, mecamylamine, dantrolene, and apoptosis detection kit Annexin V-Cy3 (AP0-AC) were obtained from Sigma (Madrid, Spain). Chelerythrine and 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) were purchased from Tocris (Biogen Científica, Spain). Sn(IV) protoporphyrin IX dichloride (SnPP) and Co (III) protoporphyrin IX dichloride (CoPP) were obtained from Frontier Scientific Europe (Lancashire, UK). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and penicillin/streptomycin were purchased from GIBC0 (Madrid, Spain). Tetramethylrhodamine ethyl ester (TMRE) and 2ℓ,7ℓ-dichlorofluorescein diacetate (H_2DCFDA) were obtained from Molecular Probes (Invitrogen, Madrid, Spain).

Culture of bovine chromaffin cells

Bovine adrenal medullary chromaffin cells were isolated as described (Livett 1984), with some modifications. Percoll gradients were omitted from the cell isolation procedure; thus, we had in our cultures a mixture of adrenergic (60–70%) and noradrenergic cells (30–40%). Cells were suspended in DMEM supplemented with 5% fetal calf serum, 50 IU/mL penicillin, and 50 **1**g/mL streptomycin. Cells were pre-plated for 30 min and proliferation inhibitors (10 μmol/L cytosine arabinoside, 10 μmol/L fluorodeoxyuridine, and 10 μmol/L leucine methyl esther) were added to the medium to prevent excessive growth of fibroblasts that would interfere with chromaffin cell death measurements; 5×10^{5} cells/well were plated in 24-well dishes. Cultures were maintained in an incubator for 2–4 days at 37°C in a water-saturated atmosphere with 5% CO_2. Cell treatments were performed in DMEM free of serum, because serum interferes with lactate dehydrogenase (LDH) measurements.

Measurement of lactate dehydrogenase activity

Samples of incubation media were collected at the end of the 24 h period of rot/oligo exposure to estimate extracellular LDH, an indication of cell death (Koh and Choi 1987; Sobrado et al. 2004). LDH activity was also measured in the cells after treatment with 10% Triton X-100 (intracellular LDH). LDH activity was measured spectrophotometrically at 490–620 nm, using a microplate reader (Labsystems iEMS reader MF; Labsystems, Helsinki, Finland). Total LDH (intracellular plus extracellular) was normalized to 100%; then, the amount of LDH released to the extracellular medium was expressed as percentage of this total. Data were normalized by subtracting basal LDH (cells not subjected to any treatment) to the different treatment groups in each individual experiment, and the result for rot/oligo group was normalized to 100% (percentage cell death).

Measurement of apoptosis and necrosis with AnnCy3/6-carboxyfluorescein diacetate

The apoptosis detection kit (AP0-AC) was purchased from Sigma, and the assay was performed according to the manufacturer's instructions. Fluorescence was measured using an inverted fluorescence microscope (Nikon eclipse TE300; Nikon Instruments Europe, Badhoevedorp, Netherlands). 6-carboxyfluorescein diacetate (6-CFDA) was excited with light at 485 nm; emitted light was transmitted through a 505 nm dichroic mirror and 520 nm emission filter; AnnCy3 was excited with light at 530 nm; emitted light was transmitted through a 575 nm dichroic mirror and 580 nm emission filter before being detected by a charge coupled device-camera.

Measurement of the mitochondrial membrane potential

To perform these experiments, bovine chromaffin cells were plated at a density of 2×10^5 cells/well in 96-well black dishes. Cells were loaded with 3 μmol/L TMRE for 15 min at 27°C in Krebs–HEPES solution (composition in mmol/L: NaCl 144, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 2, HEPES 10, and glucose 11) with 1 mmol/L probenecid. Subsequently, cells were washed twice with Krebs–HEPES solution and kept at 27°C for 15 min before the beginning of the experiment. Fluorescence was measured in a fluorescence microplate reader (FLU0star Galaxy, BMG, Germany) at 550 nm excitation and 590 nm emission wavelengths.

ROS measurement

To measure cellular R0S, we have used the molecular probe H_2DCFDA (Ha et al. 1997). Bovine chromaffin cells were loaded with 10 **1**mol/L H_2DCFDA which diffuses through the cell membrane and is hydrolyzed by intracellular esterases to the non-fluorescent form dichlorofluorescein (DCFH). DCFH reacts with intracellular H_2O_2 to form dichlorofluorescin (DCF), a green fluorescent dye. Fluorescence was measured in a fluorescence microplate reader (FLU0star Galaxy). Wavelengths of excitation and emission were 485 and 520 nm, respectively.

Immunoblotting

Chromaffin cells (5×10^6) were washed once with cold phosphate-buffered saline and lysed in 100 μL ice-cold lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mmol/L NaCl, 20 mmol/L Tris–HCl, pH 7.5, 1 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L NaF, 1 mmol/L sodium pyrophosphate, and 1 mmol/L Na_3V0_4). Protein (30 μg) from this cell lysates was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to Immobilon-P membranes (Millipore Iberica SA, Madrid, Spain). Membranes were incubated with anti-extracellular regulated kinase 1/2 (ERK 1/2; 1:1000), anti-phospho-ERK1/2 (1:1000) (Santa Cruz Biotechnology Inc., Heidelberg, Germany), anti-H0-1 (1:1000) (Chemicon, Hampshire, UK), anti-cleaved caspase 3 (1:1000) (Cell Signalling), and anti-b-actin (1:10 0000) (Sigma). Appropriate peroxidase-conjugated secondary antibodies (1:10 000) were used to detect proteins by enhanced chemiluminescence.

Image analysis and statistics

Different band intensities corresponding to immunoblot detection of protein samples were quantified using the Scion Image program (Scion Corporation, Frederick, MD, USA). Immunoblots correspond to a representative experiment that was repeated three to four times with similar results. Data are given as mean ± SEM. Differences between groups were determined by applying a one-way ANOVA followed by Newman–Keuls test. Differences were considered to be statistically significant when $p < 0.05$.

Results

Cell death induced by rot/oligo and cytoprotection afforded by epibatidine

We first setup the conditions to induce cell death by causing oxidative stress secondary to mitochondrial disruption, by blocking mitochondrial complexes I and V with the combination of rotenone plus oligomycin-A (rot/oligo). Treatment of bovine chromaffin cells for 24 h with 30 μmol/L rotenone plus 10 μmol/L oligomycin-A, transformed healthy rounded-shaped cells, isolated or grouped in clusters of two to five cells, with homogeneous birrefringent cytoplasm (Fig. 1b), into cells without birrefringency, granular cytoplasm and with many detritus (Fig. 1c). Values of LDH released, after cell exposure to a 24 h cytotoxic period of rot/oligo, are given in Fig. 1e. Basal LDH released from cells incubated with the vehicle amounted to only 5% of total; rot/oligo caused a 10-fold augmentation above basal, near 50% LDH release (black column of Fig. 1e). From now, we have normalized rot/oligo-elicited cell death as 100%.

Twenty-four hours pre-incubation with epibatidine at increasing concentrations (3–3000 nmol/L), preceding the rot/oligo period (see protocol in Fig. 1a), reduced cell death in a concentration-dependent manner (Fig. 1f). We selected the concentration of 3 μmol/L because it caused maximum cytoprotection (45%, $p < 0.001$). Pre-incubation of bovine chromaffin cells with the nicotinic agonist epibatidine (3 μmol/L, 24 h) followed by 24 h exposure to rot/oligo led to recovery of birrefringency, homogeneous cytoplasm, and round-shaped cells as shown in the microphotograph of Fig. 1d. Such cytoprotection was completely lost when cells were co-incubated with epibatidine in the presence of 30 μmol/L mecamylamine (Fig. 1g), a non-selective nicotinic receptor antagonist (Kihara et al. 1997). These data indicate that the protective effect of epibatidine was mediated by nAChR.

To determine if shorter pre-conditioning periods with epibatidine could also provide protection, bovine chromaffin cells were exposed to epibatidine for 5 min, 30 min, and 24 h; in the case of the 5- or 30-min exposures, cells were incubated in DMEM for the remaining time of the 24-h pre-incubation period (see protocol in Fig. 2a). Then, the three groups of cells were exposed to rot/oligo for 24 h. Fig. 2b shows that already after 5-min pre-incubation, epibatidine afforded 25% cell protection ($p < 0.05$). After 30 min, the protection augmented to 30% ($p < 0.05$) and was maximal (45%, $p < 0.001$) after 24 h pre-incubation, as in Fig. 1f. Although short

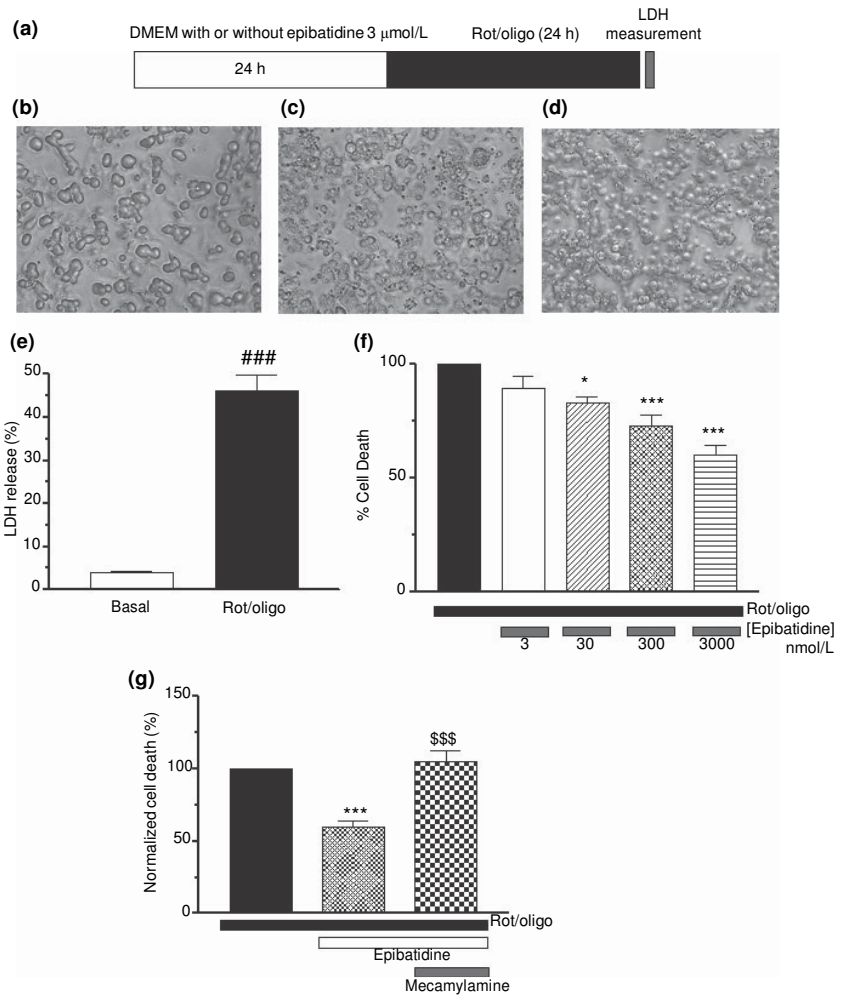

Fig. 1 Cytoprotection against cell death elicited by 30 μmol/L rotenone and 10 μmol/L oligomycin-A (rot/oligo), elicited by epibatidine, is due to nicotinic receptor activation. Panel (a) shows the protocol used to elicit cytotoxicity (24 h incubation with rot/oligo) with or without a preceding 24 h pre-incubation period with epibatidine. Panel (b) is a photomicrograph (20-) of control bovine chromaffin cells (48 h with Dulbecco's modified Eagle's medium); panel (c) shows cells exposed 24 h to rot/oligo; panel (d) shows cells pre-incubated 24 h with 3 μmol/L epibatidine followed by a 24 h incubation period with rot/oligo. Panel (e) shows the LDH released into the incubation medium in control cells (basal) and after 24 h incubation with rot/oligo; LDH (ordinate) is expressed as percentage total LDH (the sum of LDH released plus the remaining in viable cells at the experiment end). Data are mean ± SEM of 10 different cell batches. $^{###}p < 0.001$, with respect to basal. Panel (f) shows a concentration-response curve of epibatidine incubated for 24 h before rot/oligo period. Data are mean ± SEM of four different cell batches from four different cultures. $^{*}p < 0.05$ and $^{***}p < 0.001$ with respect to control cell death. Panel (g) shows the protective effect of epibatidine and its reversal by 30 μmol/L mecamylamine; here, cell death was normalized in each individual experiment, as percentage of the maximum LDH released that was considered as 100% cell death (black column). The bottom horizontal bars show cell treatments. Data are mean ± SEM of five different cell batches from five different cultures. $^{***}p < 0.001$ with respect to control cell death and $^{$$$}p < 0.001$ with respect to epibatidine.

pre-incubation times already showed cytoprotection, indicating that epibatidine-elicited an early signal that had long-lasting effects, we used the 24 h pre-incubation period in subsequent experiments to insure maximum cytoprotection.

Role of the PKC/ERK pathway in the cytoprotective effects of epibatidine

Dantrolene is a potent blocker of the ryanodine receptor channel and hence, of the CICR (Zhao et al. 2001). As illustrated in Fig. 3, we studied epibatidine cytoprotection in the absence and the presence of dantrolene (100 μmol/L), using the protocol shown in Fig. 1a. Pre-treatment with epibatidine reduced rot/oligo-induced cell death by 40%. Dantrolene completely reversed such protection. We also analyzed the involvement of PKC in the protective effect of epibatidine by using the PKC inhibitor chelerythrine (Herbert et al. 1990). When epibatidine was pre-incubated during 24 h together with 1 μmol/L chelerythrine, cytoprotection

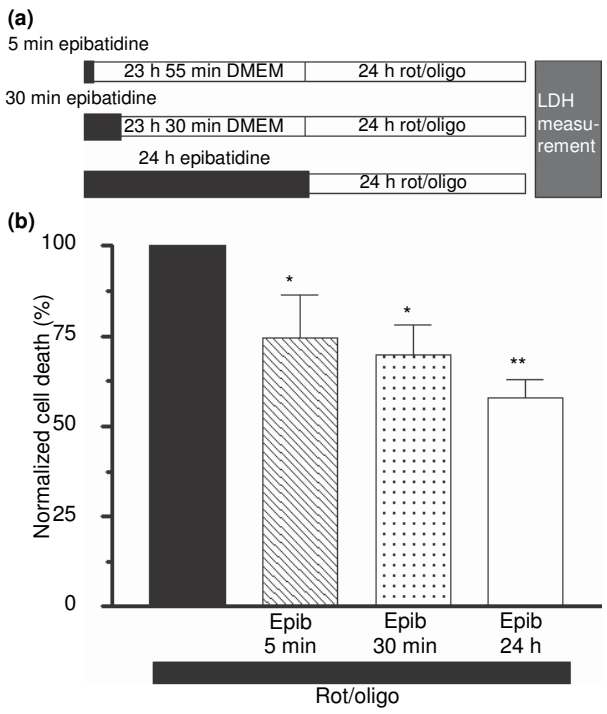

Fig. 2 Time-dependence of the cytoprotection effects of epibatidine. Cells were subjected to the protocol shown in panel (a). When cells were exposed for 5 or 30 min to epibatidine, cells were incubated with plain Dulbecco's modified Eagle's medium during the rest of the 24 h pre-incubation period. Panel (b) shows averaged data of four experiments from different cell cultures. Data are mean ± SEM. *$p < 0.05$ and **$p < 0.01$ in comparison with rot/oligo-lesioned cells in the absence of epibatidine.

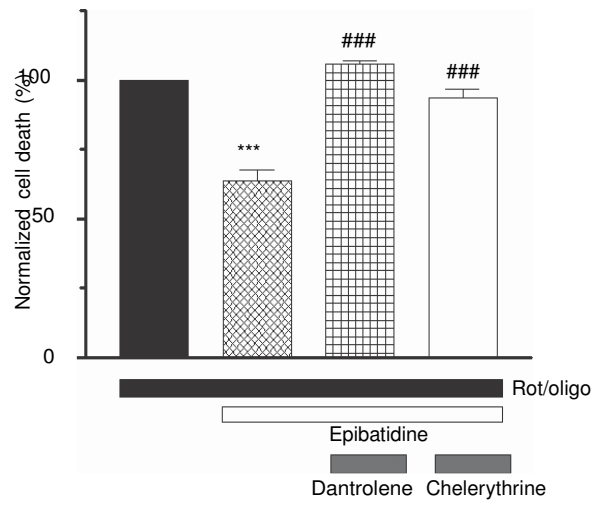

Fig. 3 Dantrolene and chelerythrine reverse the epibatidine cytoprotection. Experiments were run in parallel following the protocol shown in Fig. 1a. Cell death caused by 24 h exposure to rot/oligo was normalized to 100% (black column). Pre-incubation with 3 μmol/L epibatidine for 24 h reduced cell death by 40% (second column). If dantrolene (100 μmol/L, third column) or chelerythrine (1 μmol/L, fourth column) were present during the epibatidine pre-incubation period, cytoprotection was prevented. Data are mean ± SEM of four different cell batches. ***$p < 0.001$ with respect to control and ###$p < 0.001$ with respect to cells pre-incubated only with epibatidine.

against rot/oligo-induced toxicity was also eliminated (Fig. 3). These data suggest that ER Ca^{2+} release is participating in the long-lasting effect of epibatidine and in the activation of PKC.

Several studies have demonstrated that PKC can activate the MAP kinase cascade represented by ERK1/2 (Cox et al. 2004). Hence, we investigated the implication of ERK1/2 in epibatidine-induced cytoprotection. Figure 4a shows the western-blot of phospho-ERK1/2 and total ERK1/2. In basal conditions, phosphorylation of ERK1/2 was quite low. Pre-incubation with 3 μmol/L epibatidine for 15 min induced ERK1/2 phosphorylation to an extent similar to that of nicotine which was used as a positive control. In order to investigate the link between ERK1/2 activation and cytoprotection, cells were incubated for 24 h with epibatidine, in the absence or the presence of 50 μmol/L PD98059, a MAP kinase/ERK kinase (MEK) 1/2 inhibitor (Dudley et al. 1995), followed by another 24 h with rot/oligo (Fig. 4b). Twenty-four hours pre-incubation with epibatidine in the absence of PD98059 afforded cytoprotection against 24 h rot/oligo (45%, $p < 0.001$), while cell co-incubation with epibatidine plus PD98059 for 24 h, partially reversed this cytoprotection ($p < 0.01$). These results suggest that activation of the MEK/ERK pathway, most probably by PKC-mediated phosphorylation, is implicated in the protective effects of epibatidine.

Effects of epibatidine on the mitochondrial membrane potential and ROS production induced by the combination of rot/oligo

We used the protocol shown on top of Fig. 1 to determine if epibatidine pre-treatment could protect against mitochondrial depolarization induced by the combination of rot/oligo. At the end of the experiment, we loaded the cells, subjected to the different treatments, with 3 μmol/L TMRE (Fig. 5a); then, we measured spectrophotometrically this fluorescent probe, and finally, we measured intracellular LDH to normalize the fluorescence results in terms of viable cells. Ψmit of basal cells was normalized to 100%. After 24 h incubation with rot-oligo, Ψmit amounted to 400%; when cells were pre-incubated for 24 h with epibatidine, the Ψmit diminished to 120% ($p < 0.001$), suggesting that the nAChR triggers a signal that had a mitochondrial protecting effect.

Due to the drastic reduction of the mitochondrial membrane potential caused by pre-treatment with epibatidine, we decided to explore the existence of a possible correlation between this effect and R0S production. Hence, we measured

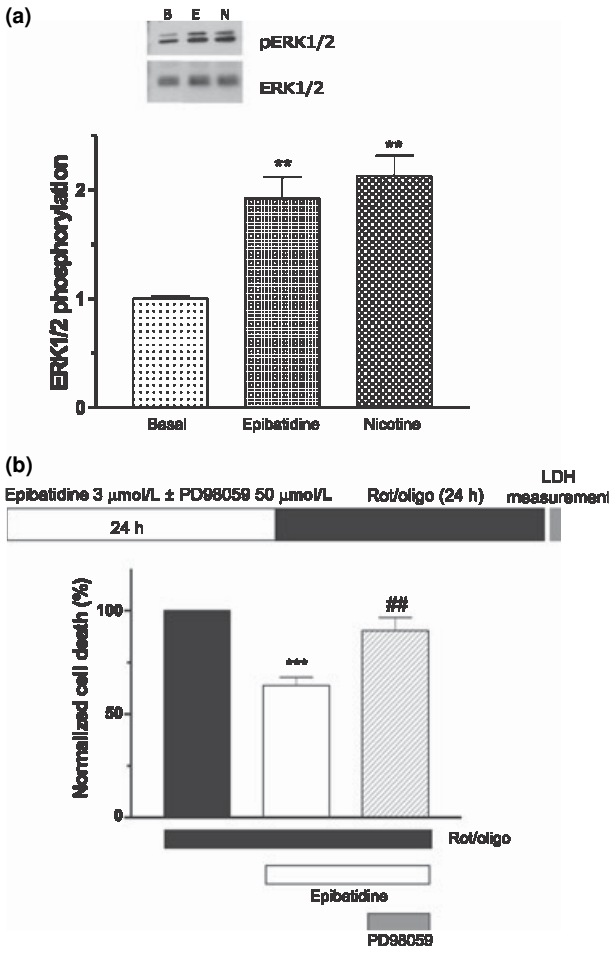

Fig. 4 Neuroprotection afforded by epibatidine against rot/oligo-induced cell death was mediated by ERK1/2 activation. (a) Immunoblot analysis of anti-phospho-ERK1/2 (Top) and anti-ERK1/2 (bottom) in bovine chromaffin cells incubated for 15 min with Dulbecco's modified Eagle's medium (Basal), epibatidine (3 µmol/L; E), or nicotine (100 µmol/L; N). The histogram expresses the densitometric quantitation of ERK1/2 phosphorylation, using total ERK1/2 for normalization. Values are mean ± SEM of three different cell batches. $^{**}p < 0.01$ with respect to control. (b) Cells were pre-incubated 24 h with epibatidine in the presence or absence of 50 µmol/L PD98059; after this pre-incubation period, cells were incubated 24 h with rot/oligo. Values are expressed as mean ± SEM of four different cell batches. $^{***}p < 0.001$ with respect to control and $^{\#\#}p < 0.01$ with respect to cells pre-incubated only with epibatidine.

R0S generation with the fluorescent probe H_2DCFDA, under the previously mentioned conditions (Fig. 1a); at the end of the 24 h period with rot/oligo, we loaded the cells with 10 µmol/L H_2DCFDA for 20 min (Fig. 5b). Spectrophotometric measurement of intracellular LDH was normalized to express the results as percentage of that in viable cells; 24 h treatment with rot/oligo increased R0S production to 220%. When cells were pre-incubated for 24 h with epibatidine, the amount of R0S produced by the cells was reduced to 130%

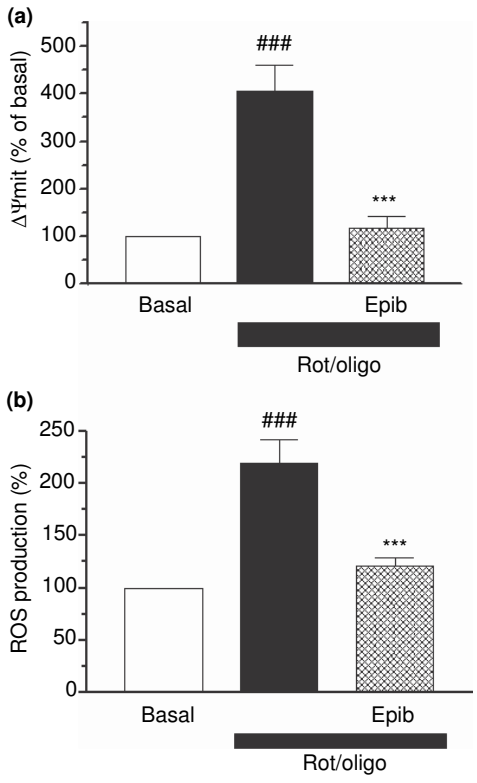

Fig. 5 Prevention by epibatidine of mitochondrial membrane depolarization (a) and intracellular reactive oxygen species (ROS) production (b) elicited by rot/oligo. Panel (a) shows the effect of 24 h pre-incubation with epibatidine on Ψmit induced by 24 h treatment with rot/oligo. (b) Pre-incubation with epibatidine reduced to near basal levels the increase of intracellular ROS production, during the 24 h treatment with rot/oligo. Values are expressed as mean ± SEM of three different cell batches. $^{***}p < 0.001$ in comparison with rot/oligo-lesioned cells in the absence of epibatidine and $^{\#\#\#}p < 0.001$ in comparison with basal.

($p < 0.001$). These results indicate that nAChR activation protect the cells when subjected to mitochondrial oxidative stress.

Epibatidine reduces apoptotic cell death induced by rot/oligo

Stabilization of the mitochondrial membrane potential and R0S production by epibatidine could be reducing apoptotic cell death induced by rot/oligo. To measure apoptotic cell death, we planned the following experiments. Cells were incubated for 24 h with or without 3 µmol/L epibatidine followed by 24 h incubation with the combination of rot/oligo and cell lysates were resolved in SDS–PAGE and analyzed with anti-cleaved caspase 3 antibody (Fig. 6a); incubation for 24 h with rot/oligo increase by 1.7-fold the amount of caspase 3 active in comparison with basal cells. Pre-incubation of the cells with epibatidine for 24 h before the rot/oligo period significantly reduced activation of caspase 3.

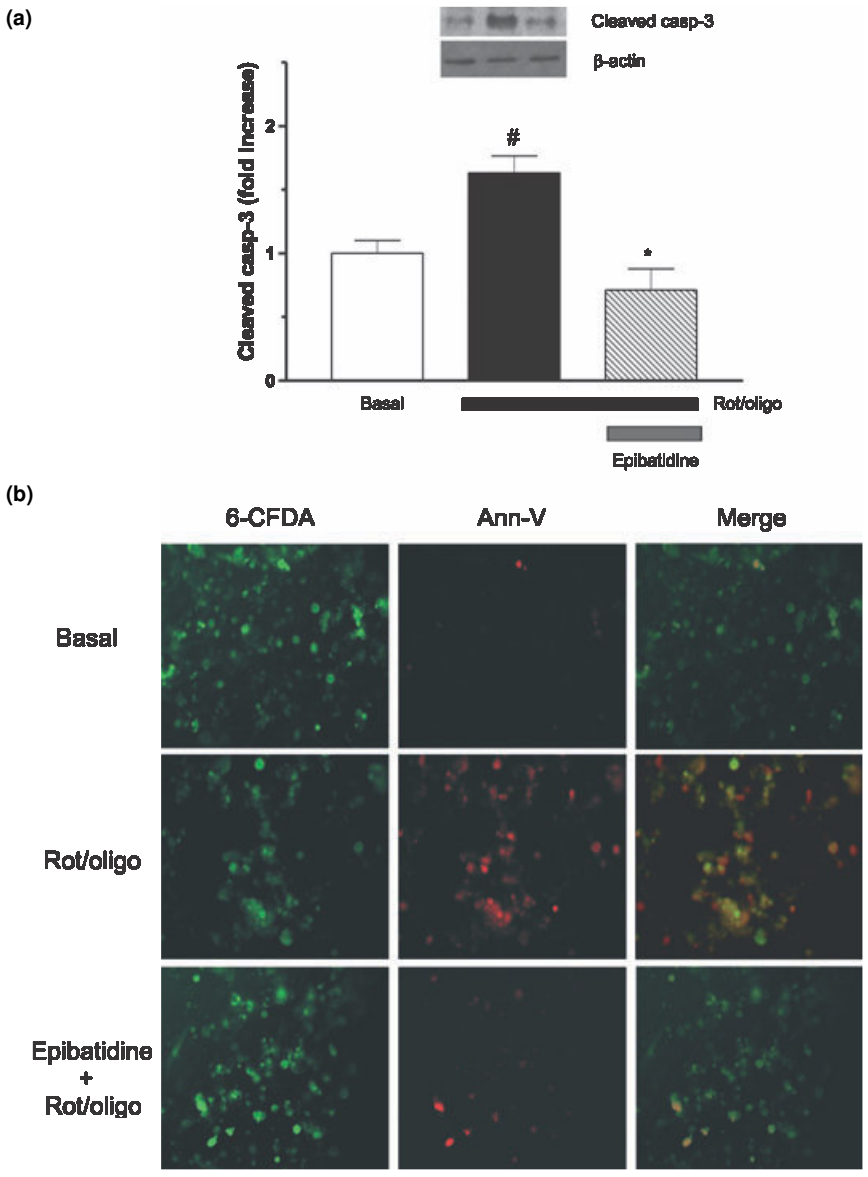

Fig. 6 Epibatidine prevents apoptosis induced by rot/oligo. (a) (Top) Immunoblot showing cleaved caspase 3 in bovine chromaffin cells untreated (track 1), incubated for 24 h with rot/oligo (track 2), and pre-incubated for 24 h with 3 μmol/L epibatidine before the rot/oligo period (track 3). Densitometric quantification of cleaved caspase 3 protein levels using b-actin for normalization is shown in the histogram. Values are expressed as mean ± SEM of three different cell batches. $^{*}p < 0.05$ in comparison with rot/oligo-lesioned cells in the absence of epibatidine and $^{\#}p < 0.05$ in comparison with basal. (b) Fluorescence photomicrographs (20·) of control cells (top), cells incubated for 6 h with rot/oligo (middle), and cells pre-incubated with epibatidine before rot/oligo treatment (bottom). The first column shows cells loaded with 6-carboxyfluorescein diacetate (6-CFDA); the middle column shows cells loaded with AnnexinV-Cy3, and the third column shows the merge of 6-CFDA and AnnCy3.

The antiapoptotic effect of epibatidine was further demonstrated in cells doubled-stained with the fluorescent dyes 6-CFDA and Annexin V-Cy3. Control cells showed viable 6-CFDA cells but hardly showed Annexin V positive cells. However, when cells were treated with the apoptotic stimulus rot/oligo for 6 h, the number of Annexin V-positive cells was markedly increased. Moreover, as they were also 6-CFDA-positive, these cells were considered apoptotic. The number of apoptotic cells was drastically reduced when cells were treated with epibatidine 24 h before the incubation with rot/oligo (Fig. 6b).

Induction by epibatidine of HO-1

For these experiments, cells were incubated for 24 h in the presence of epibatidine (3 μmol/L) and cell lysates were resolved in SDS–PAGE and analyzed by immunoblot with anti-H0-1 antibody (Fig. 7a); epibatidine increased by almost twofold the expression of H0-1, in comparison with control cells. Co-incubation of the cells with epibatidine and the MEK1/2 antagonist PD98059 reduced the over-expression of H0-1 to basal levels, indicating that ERK1/2 was implicated in the over-expression of H0-1, induced by epibatidine. Moreover, 24 h co-incubation of the cells with epibatidine, in the presence of dantrolene, also reduced H0-1 expression to basal levels.

To further analyze the involvement of H0-1 in the protective effect of epibatidine, we used SnPP, a H0-1 inhibitor (Marinissen et al. 2006), and CoPP, a H0-1 inducer (Marinissen et al. 2006). We used the protocol shown on top of Fig. 7b. Pre-incubation of epibatidine (3 μmol/L) during 24 h, followed by 24 h rot/oligo, reduced cell death by 45% ($p < 0.001$); such protection was lost when cells were

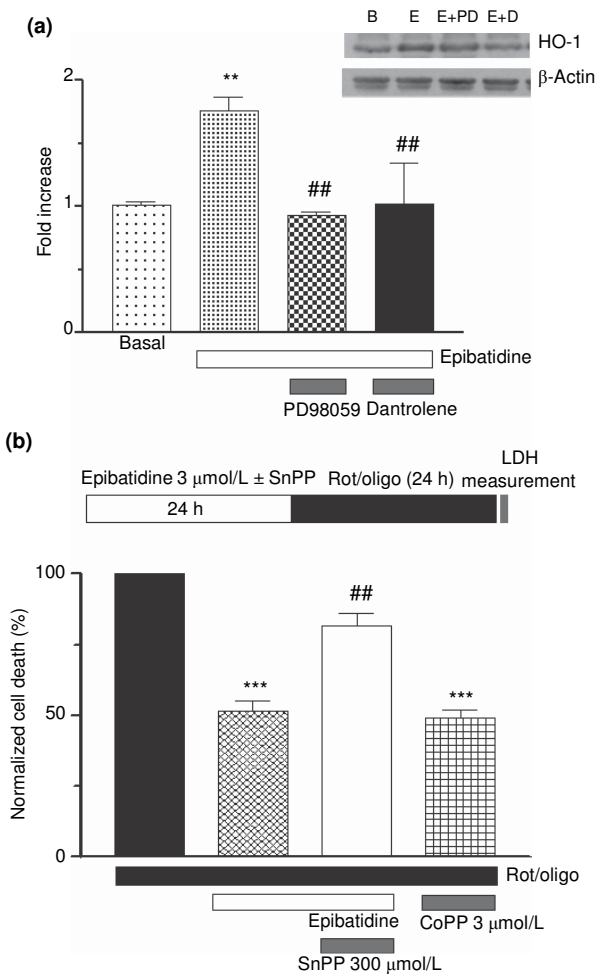

Fig. 7 Induction by epibatidine of heme oxygenase 1 (HO-1): functional link with the cytoprotective effects of epibatidine. (a) Immunoblot showing HO-1 induction in bovine chromaffin cells incubated for 24 h with epibatidine in the absence or in the presence of PD98059 or dantrolene. (Top) Blot with anti-HO-1 and anti-b-actin antibodies (B, basal; E, epibatidine for 24 h; E + PD, epibatidine plus PD98059 for 24 h; E + D, epibatidine plus dantrolene for 24 h). (Bottom) Densitometric quantitation of HO-1 protein levels using b-actin for normalization. Values are mean ± SEM of four different cell batches. $^{**}p < 0.01$ in comparison with control cells and $^{\#\#}p < 0.01$ in comparison with epibatidine-treated cells. (b) Cell death and cell protection elicited by modulation of HO-1. Cells were pre-incubated 24 h either with epibatidine alone, with epibatidine in the presence of 300 μmol/L SnPP, or with 3 μmol/L CoPP alone. After this pre-incubation period, cells were incubated 24 h with rot/oligo. Values are expressed as mean ± SEM of four different cell batches. $^{***}p < 0.001$ in comparison with rot/oligo-lesioned cells in the absence of epibatidine and $^{\#\#}p < 0.01$ in comparison with cells in the presence or the absence of SnPP.

co-incubated with epibatidine in the presence of 300 μmol/L SnPP ($p < 0.01$). Furthermore, 24 h incubation with CoPP (3 μmol/L), in the absence of epibatidine, led to cell protection against 24 h incubation with the combination of rot/oligo ($p < 0.001$). These data suggest that H0-1 participates in the protective effect afforded by nAChR activation.

Discussion

This study provides evidence for the induction of H0-1 in the neuroprotective effects of the nicotinic receptor agonist epibatidine. 0ur experiments are consistent with the following steps being involved in epibatidine protection against rot/oligo-induced death of bovine adrenal chromaffin cells.

Epibatidine opens ligand-gated neuronal nicotinic receptor channels to allow the entry of sodium and calcium ions. This causes dell depolarization to open voltage gated ion channels (i.e. sodium and/or calcium) leading to further elevation of intracellular calcium levels. Gueorguiev et al. (2004) have shown that epibatidine causes a $[Ca^{2+}]_c$ transient that promptly returned to baseline levels; however, after about 30 min the $[Ca^{2+}]_c$ slightly rose to a small plateau, suggesting that CICR was involved in such delayed effect. Using ER targeted aequorin, we have directly shown that high-K^+-evoked Ca^{2+} entry through voltage-dependent Ca^{2+} channels causes CICR in bovine chromaffin cells; furthermore, short 100-ms pulses applied to voltage-clamped cells caused a confocal cytoplasmic Ca^{2+} wave that was interrupted by ryanodine, suggesting the involvement of CICR (Alonso et al. 1999). Cell depolarization, Ca^{2+} entry and CICR, are the sequential intracellular steps resulting from epibatidine activation of nicotinic receptors, likely alpha3beta4 and alpha7 subtypes (Campos-Caro et al. 1997; Fuentealba et al. 2004) on bovine chromaffin cells. The homomeric alpha7 subtype being highly permeable to Ca^{2+} ions is a prime candidate for a $[Ca^{2+}]_c$ signal leading to epibatidine-induced cytoprotection. Although epibatidine is an agonist that causes one of the most rapid and long-lasting nicotinic receptor desensitization (Badio and Daly 1994) it caused cytoprotection; this protection was observed already after 5 min exposure (Fig. 2).

Neuronal nicotinic acetylcholine receptors agonists can activate PKC (Tuominen et al. 1992) for as long as 24 h (Tuominen et al. 1992; Bobrovskaya et al. 2007), and inhibition of PKC prevents nicotine-induced neuroprotection (Li et al. 1999). Therefore, the hypothesis emerges that ER Ca^{2+} release could maintain PKC activation along the 24 h pre-incubation period with nicotinic agonists. 0ur experimental data show that this may be so as dantrolene, a ryanodine receptor channel blocker, and chelerythrine, a PKC inhibitor, fully reversed epibatidine cytoprotection (Fig. 3). ERK1/2 is known to be phosphorylated by PKC (Cox et al. 2004) and to be responsible for mediating neuroprotection signals in various cell systems (Hetman and Gozdz 2004). In this study, epibatidine caused phosphorylation of ERK1/2 (Fig. 4a), a signal likely involved in cell protection as the MEK1/2 inhibitor PD98059 prevented such protection (Fig. 4b). This is in

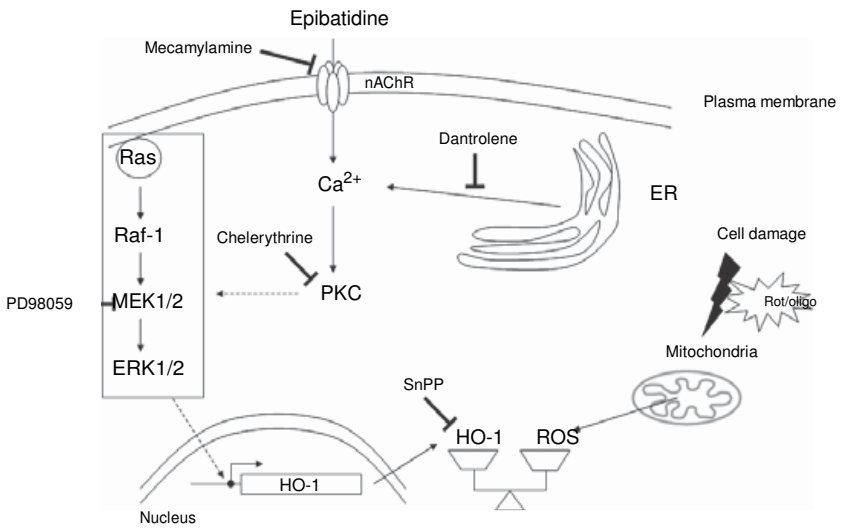

Fig. 8 Schematic representation of the neuroprotective signaling cascade activated by neuronal nicotinic acetylcholine receptors (nAChR) to induce heme oxygenase 1 (HO-1) and to prevent mitochondrial oxidative stress (see text for details).

line with recent findings showing that nicotine evokes ERK1/2 activation and neuroprotection (Dajas-Bailador et al. 2002; Toborek et al. 2007).

Heme oxygenase-1 was induced by epibatidine and its induction was prevented by PD98059 and dantrolene, indicating that Ca^{2+} from the ER and ERK1/2 are upstream signals for H0-1 induction (Fig. 7a). This was supported by the fact that SnPP (a H0-1 inhibitor) reversed epibatidine neuroprotection, and CoPP (a H0-1 inducer) produced cytoprotection by itself (Fig. 7b). ERK1/2 may up-regulate H0-1 at a 5¢-untranslated region, where several potential regulatory elements are present i.e. activator protein-1, antioxidant response elements, and GC box-binding site (Sp-1) (Elbirt and Bonkovsky 1999; Rojo et al. 2006). It may be that nicotinic receptor activation by epibatidine targets one such consensus regulatory elements to induce H0-1 and cause neuroprotection.

Two reasons determined our choice of rot/oligo to elicit cell damage and to study cell protection against such damage: (i) mitochondria are a target and a generator of R0S and are an essential component of the intrinsic apoptotic cascade (Lee et al. 2006) and (ii) the main target of the present study was H0-1, an anti-R0S protein. When mitochondria are damaged, for instance by interruption of the respiratory chain at complexes I (rotenone) and V (oligomycin-A), they are depolarized, R0S production is augmented, and a vicious circle leads to apoptosis (Fig. 6). With epibatidine, we sought to provide mitochondrial protection through: (i) maintenance of hyperpolarization and (ii) limitation of R0S production. These two beneficial effects were met by epibatidine that prevented rot/oligo-elicited mitochondrial depolarization (Fig. 5a) as well as the enhanced R0S production (Fig. 5b). Moreover, this mitochondrial protection leads to the reduction of the apoptosis induced by rot/oligo, diminishing the amount of cleaved caspase 3 (Fig. 6a).

In conclusion, we provide evidence that the neuroprotection pathway against excessive R0S production begins with nicotinic receptor activation at the cell membrane and ends with over-expression of the antioxidant enzyme H0-1 (Fig. 8). Given that excessive R0S production is involved in AD and other neurodegenerative and cerebral ischemic diseases, H0-1 activators may become good pharmacological therapies for these diseases. These potentially neuroprotective drugs may exhibit additive or synergistic effects with allosteric nicotinic receptor potentiators such as galantamine (Maelicke and Albuquerque 2000), known to have neuroprotective effects in neuronal cultures (Arias et al. 2004; Arias et al. 2005) and in hippocampal slices (Sobrado et al. 2004).

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Nrf2-mediated haeme oxygenase-1 up-regulation induced by cobalt protoporphyrin has antinociceptive effects against inflammatory pain in the formalin test in mice

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Abstract

This study investigated the effeect of haeme oxygenase-1 (HO-1) in nociception induced by formalin injection in the mice hind paw. Intraperitoneal (i.p.) administration of cobalt protoporphyrin (CoPP, an HO-1 inducer, 5 mg/kg) 24 h before the test, inhibited the nociceptive response during the second phase, but not during the first phase of the formalin test. The effect of CoPP was prevented by treatment with tin protoporphyrin (SnPP, an inhibitor of HO-1 activity) administered either by i.p. (25 mg/kg, 30 min before the test) or intraplantar (400 nmol/paw, 5 min before the test) routes. Human embryonic kidney (HEK) 293T cells treated with 10 μM CoPP expressed 20-fold higher HO-1 levels when compared to controls; this effect was suppressed by transfection with the dominant negative for the nuclear factor-erythroid 2-related factor 2 (Nrf2). Western blot analysis also revealed that CoPP treatment induced a similar 20-fold increase in HO-1 expression in the paw; this effect was attenuated in knockout mice for Nrf2. CoPP treatment of wild-type, but not in Nrf2 knockout mice, resulted in a striking increase of HO-1 stained cells surrounding the muscular tissues of the hind limbs. HO-1 positive cells were scarce in wild-type and in Nrf2 knockout untreated mice. CoPP-induced HO-1 expression in Nrf2 knockout mice was lost and correlated with the loss of antinociceptive effects. In conclusion, Nrf2-mediated HO-1 expression induced an antinociceptive effect at peripheral sites. These results suggest that HO-1 modulates the inflammatory pain pathways. Hence, the development of drugs that could raise peripheral HO-1 could be relevant in inflammatory pain treatment.

Keywords: Nrf2; HO-1; Mice; Knockout mice; Nociception; Cobalt protoporphyrin; Formalin test; Haeme oxygenase-1

1. Introduction

Haeme oxygenase (HO) is a rate limiting enzyme that degrades the pro-oxidant group haeme in three different products: iron, CO and biliverdin, which is converted into bilirubin [24]. Recently, there are a growing number of evidences that HOs are important in other physiological functions as cytoprotection in models of cellular stress [4,8], inflammation [20], and pain [10,30].

Two genetically distinct isozymes of HO have been characterised: the inducible HO-1 and the constitutive HO-2 [24]. HO-1 is typically expressed at low to undetectable levels under basal conditions in most tissues that are not involved in erythrocyte or haemoglobin metabolism, but respond to rapid transcrip-

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tional activation by diverse chemical and physical stimuli [23].

HO-2 has been implicated in nociception at the spine. Double-labelling experiments showed that a high percentage of Fos-positive nuclei identified after administration of formalin were located within HO-2 positive cells at the spine [11]. HO-2 mediates chronic inflammatory and neuropathic pain [14] and HO-2-mediated CO production seems to be related to glutamate transmission [13]. Moreover, its inhibition at this level improves the effect of morphine [12], since HO-2 mediates opioid tolerance [15].

Although there is no direct evidence for HO-1 participation in nociception, it has been suggested that CO produced by HO at the paw could limit inflammatory hyperalgesia, and that such effect is mediated by soluble guanylate cyclase activation and cGMP production [30]. It was suggested that HO-1 could be responsible for this effect since CO but not HO substrate (haeme group) reduces inflammatory hyperalgesia against PGE_2. However, their data were not conclusive on which isoform of HO was participating in this action.

Up-regulation of the HO-1 gene transcription produced by haeme or cobalt protoporphyrin (CoPP) depends on haeme-responsive elements found in the 5′-untranslated region of rodent, human, and avian HO-1 [28] where potential regulatory elements are present, i.e. activator protein-1 (AP-1) consensus, antioxidant response elements (AREs), and GC box binding site (Sp-1) [5,21]. The pathway of CoPP-dependent HO-1 induction involves at least two transcription factors, Bach1 and Nrf2, by post-transcriptional mechanisms. At lower concentrations of CoPP, Bach1 seems to be mainly responsible for the induction of HO-1, but at higher concentrations the contribution of Nrf2 increases [27].

We chose the formalin test as a model because in rat and mice intraplantar injections of formalin produce a biphasic behavioural reaction. The first phase is mostly the result of the direct stimulation of nociceptors, whereas the second phase involves a period of inflammatory sensitisation [9].

The aims of this study were to verify if CoPP-mediated HO-1 induction has an antinociceptive effect in the formalin test and if this effect is regulated by the Nrf2.

2. Materials and methods

2.1. Drugs

Formaldehyde (Merk, Darmstadt, Germany) was diluted in a saline solution (0.9% NaCl). The HO-1 inducer Co (III) protoporphyrin IX dihydrochloride (CoPP) and the HO-1 inhibitor Sn (IV) protoporphyrin IX dihydrochloride (SnPP) were obtained from Frontier Scientific, Lancashire, United Kingdom, and were dissolved with 1% DMSO in a 0.9% saline solution.

2.2. Animals

Experiments were performed in Swiss mice (25–35 g) or wild-type and Nrf2 knockout C57Bl/6J (procedent from Dr. Masayuki Yamamoto, Institute of Basic Medical Sciences and Center for Tsukuba Advanced Research Alliance, University of Tsukuba, Japan) [7]. Animals of either sex were housed under a 12-h light/12-h dark cycle (lights on at 6:00), controlled temperature and with free access to food and water. Animals (male and female mice were homogeneously distributed among groups) were acclimatised to the laboratory for at least 1 h before testing. Experiments were carried out between 9:00 and 16:00 h and the animals were used only once. The experiments were performed after approval of the protocol by the Institutional Ethics Committee and were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals [34] and all efforts were made to minimise animal suffering.

2.3. Formalin-induced nociception

The procedure used was essentially the same as that described previously [6,22,25]. Swiss mice received 20 μl of a 2.5% formalin solution (0.92% formaldehyde) made up in saline, injected intraplantar (i.pl.) in the ventral surface of the right hind paw. For C57Bl/6J mice we used a 5.0% formalin solution (1.84% formaldehyde) made up in saline in order to have the same pain response that we had with Swiss mice; differences in nociception responses between strains have also been reported in the literature [16]. Following i.pl. injection of formalin, the animals were immediately placed in a glass cylinder 20 cm in diameter, and the time spent licking the injected paw was recorded. Animals were observed from minute 0 to 5 (neurogenic phase) and from minute 15 to 30 (inflammatory phase) and the time spent licking the injected paw was recorded with a chronometer and considered as indicative of nociception.

2.4. Treatments

Protocol 1: In a first series of experiments, Swiss mice received CoPP (5 mg/kg, i.p.) or vehicle (0.9% saline solution with 1% DMSO, control animals) in a constant volume of 10 ml/kg via i.p. 24 h before being tested in the formalin test. We chose CoPP treatment to overexpress HO-1 since data from the literature support that 24 h treatment with CoPP can potently induce HO-1, without changes in HO-2 content [19].

Protocol 2: To evaluate the possible participation of HO-1 in the antinociceptive effect of CoPP in the formalin test, Swiss mice were pre-treated with CoPP (5 mg/kg, i.p.) or vehicle (0.9% saline solution with 1% DMSO, control animals) in a constant volume of 10 ml/kg via i.p. 24 h before the test. Thirty minutes before the injection of formalin, animals were treated with the inhibitor of HO-1 activity SnPP (25 mg/kg) or vehicle (0.9% saline solution with 1% DMSO, 10 ml/kg) via i.p.

Protocol 3: Alternatively, Swiss mice were pre-treated with CoPP (5 mg/kg, i.p.) or vehicle (0.9% saline solution with 1% DMSO, control animals) in a constant volume of 10 ml/kg via i.p. twenty-four hours before the test. 5 min before the injection of formalin, animals were treated with SnPP (288.1 μg/20 μl or 400 nmol/20 μl/paw as referenced in the figures and Section 3) or vehicle (0.9% saline solution with 0.1% DMSO, 20 μl/paw) at the same paw of the formalin injection.

Protocol 4: In order to verify the participation of the transcription factor Nrf2 in the nociceptive mechanisms of CoPP, we used Nrf2 knockout mice. Both C57Bl/6J wild-type and Nrf2 knockout mice were treated with CoPP (5 mg/kg, i.p.) 24 h before formalin test.

2.5. Cell culture

Human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 80 μg/ml gentamycin.

2.6. Luciferase assays

Transient transfections of HEK293T cells were performed with the expression vectors ARE-LUC (Dr. J. Alam, Department of Molecular Genetics, Ochsner Clinic Foundation, New Orleans, LA) or the promoter-less control vector pGL3b (Promega). Then, cells were co-transfected either with the empty vector (pCDNA3 vector) or pEF-DNrf2(DN) (Dr. J. Alam, Department of Molecular Genetics, Ochsner Clinic Foundation, New Orleans, LA). HEK293T cells were seeded in 24-well plates (75,000 cells per well), cultured for 16 h and transfected with calcium phosphate, using the reagents from Sigma–Aldrich (Madrid, Spain). The cells were treated with 10 μM CoPP or 30 μM SnPP in low-serum medium (0.5% fetal bovine serum) for 16 h, lysed and assayed for luciferase activity with the Luciferase Assay System (Promega), according to the manufacturer's instructions. Relative light units were measured in a BG1 OptocompI, GEM Biomedical luminometer (Sparks, NV). Basal luminescence values obtained in pGL3b transfected cells with respective treatments (including pCDNA3 vector or pEF DNrf2(DN) transfection) were subtracted from ARE-LUC transfected cells plus treatments.

2.7. Western blot analysis

After the formalin test, mice were sacrificed by cervical dislocation and the contralateral formalin non-injected tissues of the hind paws were homogenated in 100 μl ice-cold lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris–HCl, pH 7.5, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, and 1 mM Na_3VO_4). Protein (30 μg) from these cell lysates was resolved by SDS–PAGE and transferred to Immobilon-P membranes (Millipore Corp.). Membranes were incubated with anti-HO-1 (1:1000) (Chemicon); anti-β-actin (1:100,000) (Sigma). Appropriate peroxidase-conjugated secondary antibodies (1:10,000) were used to detect proteins by enhanced chemiluminescence.

2.8. Immunohistochemistry and analysis of HO-1

C57Bl/6J wild-type and knockout mice treated either with CoPP or vehicle were anaesthetised immediately after the formalin test with 3% isoflurane in oxygen under spontaneous respiration. Then, the animals were sacrificed by perfusion with saline solution followed by fixation with freshly prepared 4% paraformaldehyde in 0.1 mol/l phosphate buffer pH 7.4. The hind limb muscle tissue was embedded in paraffin prior to sectioning on a rotary microtome (Shandon AS-325-Retraction) in 5 μm sections that were used for immunohistochemistry.

After being deparaffinised and rehydrated, the muscular tissues of the hind limbs were immersed in 0.01 mol/l citrate pH 6.0 buffer and boiled for 20 min. Sections were then blocked with 1.5% bovine serum albumin and incubated for 48 h with the primary antibody (anti-haeme oxygenase-1, Chemicon, 1:50). Sections were immersed in 3% hydrogen peroxide in methanol and incubated with a secondary anti-rabbit antibody conjugated with peroxidase (1:200) followed by 0.06% diaminobenzidine, 0.2% hydrogen peroxide. Negative control sections were incubated without the primary antibodies. Sections were counterstained with haematoxylin.

2.9. Statistical analysis

Comparisons between experimental and control groups were performed by unpaired t test or two-way ANOVA followed by Newman–Keuls test when appropriate. A value of $P < 0.05$ was considered to be significant.

3. Results

3.1. Effect of treatment of the animals with CoPP in the formalin test

To evaluate if HO-1 induction could afford an antinociceptive effect in mice injected with formalin in the paw, the animals were treated with CoPP 5 mg/kg, i.p. 24 h before testing (protocol 1; Section 2.4). In parallel, a control group was injected i.p. with saline. The time the animals spent licking the paw injected with formalin during the first phase of the test was 112.2 ± 8.8 s and 100.0 ± 7.3 s for the control and CoPP groups, respectively (Fig. 1a); the difference between these values was not statistically significant (unpaired t test, $P = 0.679$) (Figs. 1a and 1b). However, when the animals were evaluated during the second phase of this test, licking time of the formalin injected paw was reduced from 108.05 ± 22.04 s in saline treated mice to 57.4 ± 23.0 s in mice treated with CoPP at minute 20 and from 121.4 ± 11.1 to 72.1 ± 16.0 at minute 25 of the test (unpaired t test, $P < 0.05$). However, at minute 30, the time the animals spent licking the paw was not statistically significant between both groups (Fig. 1a). Therefore, these results suggested that HO-1 induction by CoPP caused around 45% reduction of nociception during the second nociceptive phase induced by formalin (Fig. 1c).

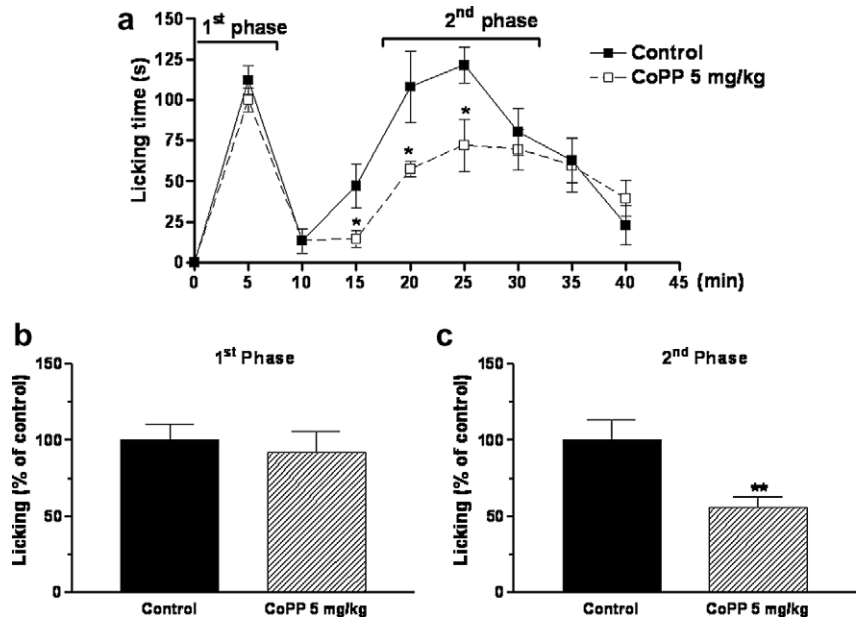

Fig. 1. (a) Time course of the formalin test in control and in CoPP treated mice. Animals were injected in the paw with 20 μl of a 2.5% formalin solution immediately before the test. Values represent means ± SEM of 8 animals. (b and c) The antinociceptive effect of treatment of Swiss mice with CoPP (5 mg/kg, i.p.) 24 h before the formalin test. Values are normalised taking the mean of the control group as 100%; they are expressed as mean ± SEM and represent the licking time in the first phase (b) and second phase (c) of the formalin test. (n = 5–8), unpaired t test $^{*}P < 0.05$ and $^{**}P < 0.01$ vs vehicle-treated control.

3.2. Inhibition of HO-1 by treating the animals with SnPP prevented the antinociceptive effects of CoPP

During the first phase of the formalin test, the vehicle, CoPP, SnPP or CoPP + SnPP groups treated by the i.p. route (protocol 2; Section 2.4) were not statistically different (data not shown). A two-way ANOVA showed that there was no effect of CoPP pre-treatment ($P = 0.318$), SnPP treatment ($P = 0.114$) nor pre-treatment · treatment interaction ($P = 0.734$).

Fig. 2 shows the effect of the treatment of Swiss mice with vehicle, CoPP, SnPP i.p., or CoPP + SnPP i.p. during the second phase of the formalin test (see Section 2.4; protocol 2 for treatments). The post hoc analysis

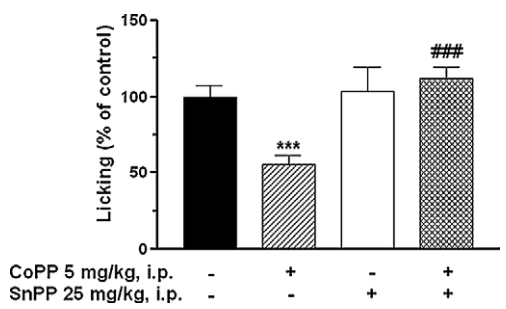

Fig. 2. Effect of the pre-treatment of Swiss mice with SnPP (25 mg/kg, i.p.), on CoPP (5 mg/kg, i.p.)-induced antinociception in the formalin test in mice. Each column represents the mean ± SEM (n = 5–10). A two-way ANOVA followed by a Newman–Keuls test $^{***}P < 0.001$ as compared with the vehicle-treated control. $^{###}P < 0.001$ as compared with the CoPP group.

showed a significant antinociceptive effect for CoPP ($P < 0.001$) and SnPP, administered by i.p. route 30 min before the test, completely reverted the effect of CoPP in the second phase of the formalin test ($P < 0.001$).

In the experiments where SnPP was injected by i.pl. route, to revert the effect of CoPP injected 24 h before the test (protocol 3; Section 2.4), no difference was found between control, CoPP, SnPP or CoPP + SnPP groups during the first phase of the formalin test (data not shown). A two-way ANOVA showed that there was no effect of CoPP pre-treatment ($P = 0.367$), SnPP treatment ($P = 0.289$) nor pre-treatment · treatment interaction ($P = 0.638$).

Fig. 3 shows the effect of the treatment of Swiss mice with vehicle, CoPP, SnPP i.pl., or CoPP + SnPP i.pl. during the second phase of the formalin test (see Section 2.4; protocol 3 for further details). The post hoc analysis showed a significant antinociceptive effect of CoPP ($P < 0.001$). SnPP administered by i.pl. route 5 min before the test completely reverted the effect of CoPP in the second phase of the formalin test ($P < 0.001$). These results suggested that HO-1 was being induced locally at the paw and was responsible for the antinociceptive effect during the second phase of the formalin test.

3.3. HO-1 induction by CoPP was related to the transcription factor Nrf2

HEK293T cells were transiently transfected with ARE-LUC or with a control promoter-less vector

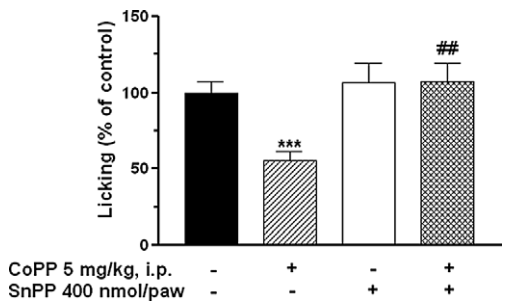

Fig. 3. Effect of the pre-treatment of Swiss mice with SnPP (400 nmol/20 μl/paw), on CoPP (5 mg/kg, i.p.)-induced antinociception in the formalin test. Each column represents the mean ± SEM (n = 6–10). Two-way ANOVA followed by Newman–Keuls test $^{***}P < 0.001$ as compared with the vehicle-treated control. $^{\#\#}P < 0.01$ as compared with the CoPP group.

(pGL3basic) and treated with 10 μM CoPP or 30 μM SnPP for 16 h. As shown in Fig. 4a, CoPP treatment, but not SnPP, induced 20-fold increase in luciferase activity compared with pCDNA3 vector transfected cells. To identify the transcription factor implicated in the induction of the ARE system of the ho-1 promoter, we co-transfected HEK293T cells with reporter plasmids ARE-LUC or pGL3basic and a expression vector for Nrf2 dominant negative version, pEFDNrf2(DN). Nrf2 activity was blocked by overexpression of the bZIP dimerisation domain of Nrf2 (DNrf2(DN)) that competes with endogenous Nrf2 for heterodimerisation. In Fig. 4a we show that the activation of the promoter by CoPP was completely blocked in pEFDNrf2(DN) co-transfected cells. Taken together these results suggest that CoPP induces HO-1 expression by the regulation of antioxidant response elements (ARE) in an Nrf2 dependent manner.

To verify that CoPP treatment was indeed overexpressing HO-1 via Nrf2, we observed the plantar surface of the contralateral paws of the Nrf2 +/+ and −/− mice treated or untreated with CoPP after the formalin test and quantified HO-1 by Western blot analysis and by immunostaining. A two-way ANOVA showed significant differences for CoPP ($P < 0.001$), Nrf2 presence ($P < 0.05$) and interaction ($P < 0.05$). As represented in Fig. 4b, a post hoc analysis shows that treatment of the mice with a single dose of 5 mg/kg CoPP, i.p. also raises HO-1 expression by about 20-fold when measured 24 h after treatment ($P < 0.01$). The same treatment in knockout mice for Nrf2 showed only fourfold increase in HO-1 expression ($P = 0.254$).

As seen by the Western blot technique, we verified that HO-1 staining was increased in the muscle sections of the hind paw when analysed by immunohistochemistry in wild-type mice treated with CoPP when compared with control animals. HO-1 staining was almost absent in Nrf2 knockout mice treated with saline, and scarce in animals treated with CoPP (Fig. 5).

3.4. The antinociceptive effect of HO-1 induction by CoPP was abolished in Nrf2 knockout mice

Once we had proven that CoPP induces HO-1 via Nrf2, we used Nrf2 knockout mice to determine if the antinociceptive effect of HO-1 was related to the transcription factor Nrf2 (see Section 2.4; protocol 4). The results of these experiments are represented in Fig. 6. In C57Bl/6J wild-type mice, we observed a similar pat-

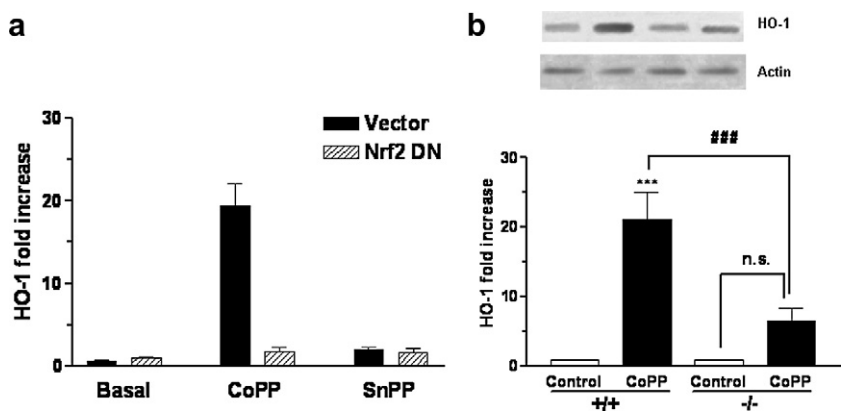

Fig. 4. (a) Dominant negative Nrf2 mutant prevents the up-regulation of the ho-1 promoter by CoPP. HEK293T cells were co-transfected with a luciferase reporter construct carrying the 15-kb of the 5′-promoter region of the mouse HO-1 gene (p15-LUC) or with the promoter-less control vector (pGL3b) plus the empty pCDNA3 vector or the dominant negative version of Nrf2 (DNrf2(DN)). After 24 h from transfection, cells were treated with 10 μM CoPP or 30 μM SnPP for 16 h. Results in graphics represent means ± SEM of two independent experiments. (b) Western blot showing the expression of HO-1 in animals, treated with CoPP (5 mg/kg, i.p.) or vehicle for 24 h, after the formalin test. (Lane 1) Wild-type animals without CoPP treatment. (Lane 2) Wild-type animals treated with CoPP. (Lane 3) Nrf2 knockout mice without CoPP treatment. (Lane 4) Nrf2 knockout mice treated with CoPP. b-actin is showed as a second blot below HO-1. Results in graphics represent the mean ± SEM of two independent experiments. Two-way ANOVA followed by Newman–Keuls test $^{***}P < 0.001$ vs vehicle-treated control. $^{\#\#\#}P < 0.001$ vs wild-type treated with CoPP.

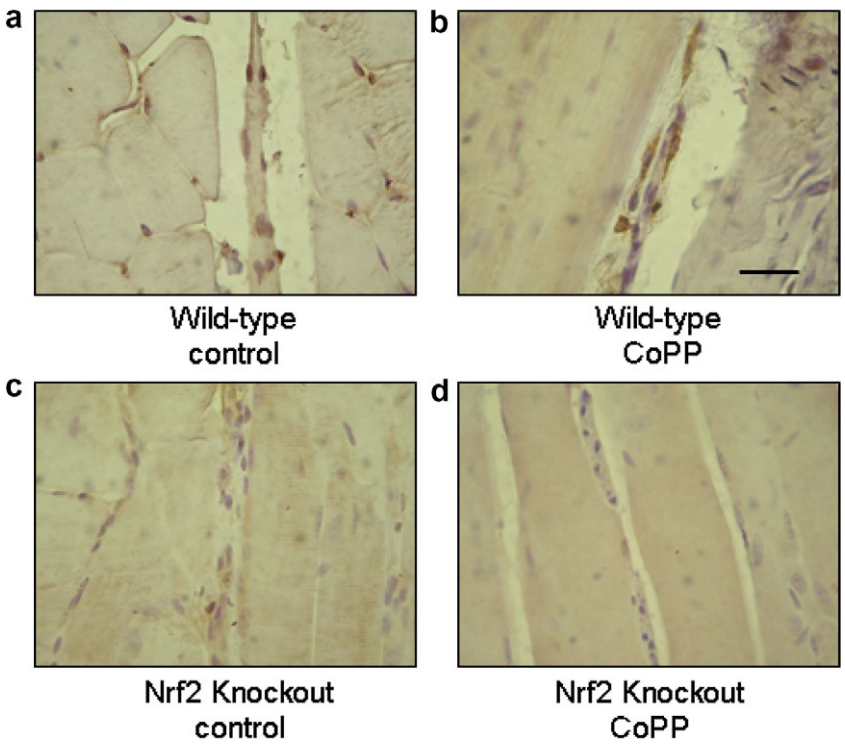

Fig. 5. Immunohistochemistry for HO-1 in contralateral limb muscle sections of C57B1/6J after the formalin test. (a) Wild-type animals without CoPP treatment; (b) wild-type treated with CoPP (5 mg/kg, i.p., 24 h); (c) Nrf2 knockout without CoPP treatment or (d) Nrf2 knockout treated with CoPP. Positive staining for HO-1 is seen as brown marked cells, all tissues were counterstained with haematoxylin.

tern as in Swiss mice; CoPP (5 mg/kg, i.p.) did not modify licking time during the first phase but significantly reduced this value during the second phase of the formalin test. However, in Nrf2 knockout mice, the antinociceptive effect of CoPP in the second phase of the formalin test was completely lost, indicating that this transcription factor mediates the antinociceptive effects of HO-1 induction. The two-way ANOVA showed significant differences for CoPP treatment ($P < 0.001$), Nrf2 presence ($P < 0.001$) and interaction ($P < 0.05$). As illustrated in Fig. 6, a post hoc analysis shows that treatment of the mice with a single dose of 5 mg/kg

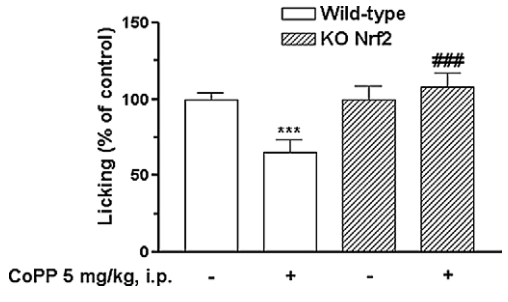

Fig. 6. Effect of the treatment with vehicle or CoPP (5 mg/kg, i.p.), 24 before the formalin test, in wild-type and knockout mice for Nrf2 (KO Nrf2). Values are expressed as mean ± SEM and represent the licking time in the second phase of the formalin test ($n = 5–8$). $^{***}P < 0.01$ vs vehicle-treated control. $^{\#\#\#}P < 0.001$ vs wild-type treated with CoPP.

CoPP produced an antinociceptive effect in the wild-type mice ($P < 0.001$). The absence of Nrf2 protein prevented the effect of the treatment with CoPP ($P < 0.001$).

4. Discussion

In this paper we have shown that the overexpression of HO-1 through the transcriptional factor Nrf2, in animals treated with CoPP, can modulate pain behaviour.

The antinociceptive effect of HO-1 induction with CoPP was effective during the second but not during the first phase of the formalin test. These results indicate that HO-1 is mainly participating in preventing nociception of an inflammatory origin [9,22]. This is consistent with the profuse literature showing the participation of HO-1 in inflammation [17,20,26,33]. Two of the most significant facts that point out the importance of HO-1 in inflammation are that HO-1-deficient mice develop a chronic inflammatory state that progresses with age [20] and that the only human reported to lack HO-1 enzymatic activity died of an inflammatory syndrome [33]. This, together with the fact that the expression of HO-1 is increased in inflammatory diseases [17,26], could mean that HO-1 participates as a limiting anti-inflammatory agent.

The antinociceptive effect elicited by CoPP treatment seems to be related to the HO-1 isoform of the enzyme. This was supported by (i) the fact that CoPP treatment

24 h before the formalin test via i.p. enhanced 20-fold the expression of HO-1 in the paw and (ii) SnPP, an inhibitor of HO, injected 30 min before the test prevented the antinociceptive effect of CoPP during the second phase of the formalin test. Although SnPP inhibits both HO isoforms, it only seems to modulate nociception when HO-1 is overexpressed in CoPP treated animals. Further data that support that HO-1 is responsible for the antinociceptive action is that SnPP, at least at the doses tested in this study, did not modify nociception per se in any of the phases of the test, suggesting that the constitutive HO-2 does not seem to be participating in peripheral modulation of nociception described here. Furthermore, it was recently described that CoPP selectively enhances HO-1, but not HO-2 [19].

There are studies in the literature suggesting that HO at the periphery could have a modulatory effect in nociception in rats. The administration of haeme, an HO substrate, in the paws inhibited hyperalgesia induced by IL-1β; however, when the inflammatory agent was PGE_2, which does not overexpress HO-1, the effect of haeme was not observed [30]. More recently, it was suggested again that HO acting at the periphery had antinociceptive effects through the CO-cGMP pathway in the formalin test. In this case, the observed effect was most probably not due to inducible HO-1 since they did not utilise any approach to overexpress HO-1.

ZnPP, which inhibits both isoforms of HO, produced a pro-nociceptive effect in the formalin test [18]. However, in our study we show that treatment of the animals with SnPP locally at the paw reverts the effect of HO-1 overexpression by CoPP treatment in the formalin test, clearly indicating that HO-1 is acting at the periphery to inhibit nociception. Moreover, we have seen an increase in HO-1 staining in C57Bl/6J wild-type mice treated with CoPP compared to untreated animals. The positive HO-1 staining was predominantly observed in conjunctive tissue and around muscle tissues.

The natural substrate of HO-1, haeme, serves as an alert signal during tissue injury. When locally released, it can function as a physiological trigger to start inflammatory processes. Haemin, the oxidised form of haeme, produces a concentration dependent induction of HO-1 gene transcription, with a maximal induction around 20-fold [2,29], value that corresponds exactly with the induction exerted by CoPP in our work. On the other hand, HO-1 inhibits inflammation, down-regulating adhesion molecules expression and subsequently reducing the binding of leukocyte as a later feedback mechanism [32]. It is interesting to think that, as suggested by our results, HO-1 could also act as a physiological modulator of inflammatory pain, inhibiting the painful consequences of a noxious stimulus that has the opposite effect of HO-2 in pain pathways [10,13].

Nrf2 is one of the transcription factors responsible for HO-1 induction by haeme [1]. Interestingly, Nrf2 has also been implicated in healing wounding process, it seems that Nrf2 is activated in early phases of wounding by the reactive oxygen species generated [3]. CoPP has also been shown to raise HO-1 expression through the transcription factor Nrf2 [27]. To study if induction of HO-1 by CoPP depends on Nrf2, we first confirmed in HEK cells that HO-1 expression depends on this transcription factor. After that, we confirmed by Western blot analysis that Nrf2 knockout animals have approximately fivefold less capacity to overexpress HO-1 when injected with CoPP. These data were also confirmed by the small amount of positive HO-1 cells in Nrf2 knockout mice treated with CoPP. This loss of HO-1 induction by CoPP treatment in Nrf2 knockout mice correlated with the loss of nociception in the second (inflammatory) phase of the formalin test. This finding is related to the observation that Nrf2 protects against inflammation in animal models and is supported by the observation that Nrf2 knockout mice are more susceptible to lethal doses of lipopolysaccharide or that cytokine and chemokines production induced by lipopolysaccharide are higher in these animals [31].

In summary, our data show that HO-1 modulates the inflammatory pain pathways and that its overexpression is implicated in the antinociceptive effect of CoPP in mice. We also show that the effect of CoPP depends on Nrf2. Therefore, therapeutic strategies involving HO-1 induction could be considered as a new target for analgesia.

Acknowledgments

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Haeme oxygenase-1 overexpression in white blood cells via α7 nAChRs and the trancription factor Nrf-2 has antinociceptive effects in the formalin test

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(Enviado a *Pain*, en evaluación)

Abstract

Epibatidine has shown antinociceptive effects in various pain models, being 200-fold more potent than morphine. Furthermore, epibatidine is capable to induce haeme oxygenase-1 (HO-1). On the other hand, previous results of our laboratory demonstrate that HO-1 over-expression has antinociceptive effect in the formalin test. So, the aim of this study was to investigate the effect of HO-1 over-expression induced by epibatidine in nociception elicited by formalin injection in the mice hindpaw. Epibatidine administration (4 μg/kg) 24 h before the test reduced the nociceptive response during the first and second phase of the formalin test. This effect was prevented by treatment with tin protoporphyrin (SnPP, an inhibitor of HO-1 activity) administered via intraplantar 5 min before the test, suggesting a peripheral role of HO-1. Western blot analysis revealed that epibatidine treatment increased by 2-fold HO-1 expression in the paw; this effect was lost in knockout mice for nuclear factor-erythroid 2-related factor 2 (Nrf2) and was accompanied with the loss of antinociceptive effects. Furthermore, the antinociceptive effect of epibatidine was related with the activation of the α7 nicotinic receptors, since methyllycaconitine (MLA) revert this effect. Finally, we showed by flow cytometry and by immunofluorescence that white blood cells of the animals injected with epibatidine expressed more HO-1 than control animals, and this expression was reverted by MLA pre-treatment. These findings demonstrate that HO-1 induction by epibatidine has antinociceptive and anti-inflammatory effects at peripheral sites via α7 nAChR activation in white blood cells.

1.Introduction

Epibatidine, an alkaloid isolated from the skin of the Equatorian poison-arrow frog *Epipedobates tricolour*, was found to be a potent non-opioid antinociceptive compound [1], 200-fold more potent than morphine. Epibatidine has shown antinociceptive effects in various models of pain such as the tail-flick test, hot plate test [2], and formalin test in the hind paw [3] and in the orofacial versions [4]. Moreover, epibatidine has shown potent antinociceptive activity in some acute and chronic pain models when administered intrathecally or intraspinally in rats [5] or mice [6; 7], and suppressing hyperalgesia and allodynia induced by nerve injury and capsaicin [8]. The antinociceptive mechanism of action of epibatidine remains unclear but is believed to be related with the activation of neuronal nicotinic acetylcholine receptors (nAChRs) since mecamylamine, but not naloxone, an opioid antagonist, reverts its antinociceptive effects [9].

nAChRs subtypes are involved in different physiological functions, depending on their location in tissues [10; 11]. Hence, there are evidences that point to the α4β2 nAChRs as the key receptor in antinociception in central nervous system (CNS) [12; 2; 13; 14]. On the other hand, α7 nAChRs, which are distributed extensively in CNS and peripheral sites, are involved in many important functions [15-17], including neuroprotection [18] and antinociception. In fact, choline, the precursor of acetylcholine (ACh), which is a selective endogenous agonist for α7 nAChRs [19], has antinociceptive effects against acute and inflammatory pain [20]. Furthermore, α7 nAChRs were localized in the plasma membrane of lymphocytes and macrophages and have been proposed as an essential regulator of inflammation [21].

Haeme oxygenase (HO) is the rate-limiting enzyme that degrades the pro-oxidant heme group and produces equimolecular quantities of carbon monoxide, iron, and biliverdin. Biliverdin is subsequently reduced to bilirubin by biliverdin reductase. These three by-products have been related to cell protection [22; 18], inflammation [23], and pain [24; 25]. There are two isoforms of this enzyme, HO-1 (inducible isoform) and HO-2 (constitutive isoform). The pathway of cobalt protoporphyrin (CoPP)-dependent HO-1 induction involves at least two transcription factors, Bach1 and Nrf2, by post-transcriptional mechanisms. At lower concentrations of CoPP, Bach1 seems to be the main responsible for the induction of HO-1, but at higher concentrations the contribution of Nrf2 increases [26].

This study is based on two observations of our laboratory: i) we have recently shown that HO-1 induction by CoPP has an antinociceptive effect in the second phase of the formalin test and that it depends on Nrf2 [27]. This effect was related to anti-inflammatory actions of HO-1; and ii) We have shown that 24 h pre-incubation with epibatidine is capable of inducing HO-1 *in vitro* [18]. Therefore, the aims of this study were to verify if HO-1 induction by 24 h pre-treatment with epibatidine could have an antinociceptive effect in the formalin test and if this effect is related to peripheral alpha7 nAChRs.

2.Materials and Methods

Drugs

Formaldehyde (Merk, Darmstadt, Germany) was diluted in saline solution (0.9% NaCl). Epibatidine hydrochloride, mecamylamine hydrochloride and methyllycaconitine hydrochloride were purchased from Sigma (Madrid, Spain), and the HO-1 inhibitor Sn (IV) Protoporphyrin IX dihydrochloride (SnPP) was obtained from Frontier Scientific (Lancashire, United Kingdom).

Animals

Experiments were performed in male *Swiss* mice (25-35 g) or wild-type C57JBl/6J and Nrf2 knockout mice [28]. Animals were housed under a 12-h light/12-h dark cycle (lights on at 6:00), controlled temperature and with free access to food and water. Animals were acclimatised to the laboratory for at least 1 h before testing. Experiments were carried out between 9:00 and 16:00 h and the animals were used only once. The experiments were performed after approval of the protocol by the Institutional Ethics Committee, in accordance with the law in force (European Directive 86/609/EEC and Real Decreto 1201/2005), following the Research Council's Guide for the Care and Use of Laboratory Animals and the ethical guidelines for investigations of experimental pain in conscious animals. All effort was made to minimise animal suffering.

Formalin test and treatments

The procedure used was the same as that described previously [29]. Briefly, animals were observed from 0-5 min (neurogenic phase) and 15–30 min (inflammatory phase). The time spent licking the injected paw was recorded with a chronometer and considered as indicative of nociception. Epibatidine 4 μg/kg was injected via i.p. 24 h before the formalin test and the HO-1 inhibitor SnPP was injected via i.pl. (400 nmol/paw) 5 min before the test to ensure the inhibition of HO-1. MLA (0.2 mg/kg) and mecamylamine (2 mg/kg) were injected via i.p. 30 min before the injection of epibatidine to ensure the blockade of nicotinic acetylcholine receptors.

Western blot analysis

After the formalin test, mice were sacrificed by cervical dislocation and the contralateral formalin non-injected tissues of the hind paws were homogenated in 100 μl ice-cold lysis buffer (1% Nonidet P-40, 10% glycerol, 137 mM NaCl, 20 mM Tris–HCl, pH 7.5, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM sodium pyrophosphate, and 1 mM Na_3VO_4). Protein (30 μg) from this cell lysates was resolved by SDS–PAGE and transferred to Immobilon-P membranes (Millipore Corp.). Membranes were incubated with anti-HO-1 (1:1000) (Chemicon); anti-・-actin (1:100000) (Sigma). Appropriate peroxidase-conjugated secondary antibodies (1:10000) were used to detect proteins by enhanced chemiluminescence.

Flow cytometry

After the spleen were removed aseptically and placed in Petri dishes containing sterile supplemented medium (RPMI 1640), the cells were gently teased apart. Following the removal of clumps by centrifugation, the cells were suspended in RPMI 1640, containing 5 % heat-inactivated fetal calf serum, 0.02 mM 2-mercaptoethanol and gentamicin (50 mg/mL), and were counted. For this study, we used the following specific monoclonal antibodies: Rat anti-mouse CD16/CD32 (Mouse Fc Block) (1:50; BD Pharmingen, San Diego, CA, USA), anti-HO-1 (1:50; Chemicon, Hampshire, UK) and goat anti-rabbit IgG Alexa Fluor 568 (1:250; Invitrogen, Carlsbad, CA).

Hematoxylin-eosin, immunohistochemistry and immunofluorescence of HO-1 and F 4/80-like-receptor (FIRE)

Mice spleens were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, embedded in paraffin and sectioned on a rotary microtome (Shandon AS-325-Retraction, Thermo Electron Corporation, Waltham, MA) in 5-μm sections that were stained with hematoxylin-eosin or used for immunohistochemistry and immunofluorescence as follows. After deparaffination and rehydration, sections were immersed in 3% H_2O_2 in methanol and then in 0.01 M citrate buffer, pH 6.0, and boiled for 20 min. Sections were then blocked with 10% normal goat serum and

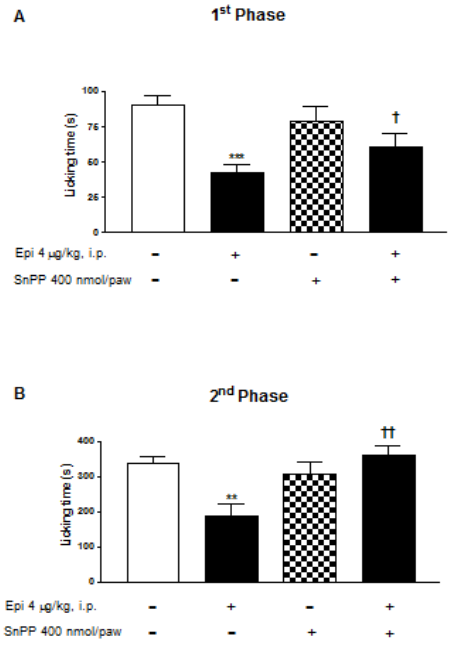

Figure 1. The antinociceptive effect of epibatidine, injected 24 h before the test, was reverted by pretreatment with SnPP. Effect of the pretreatment of *Swiss* mice with SnPP (400 nmol/20 µl·paw), on epibatidine (4 µg/kg, i.p.)-induced antinociception in the first (A) and second phase (B) of the formalin test. Data represent means ± SEM (n = 8-10). Two-way ANOVA followed by Newman-Keuls test. $^{**}p<0.01$ and $^{***}p<0.001$ compared with the vehicle-treated control. $^{\dagger}p<0.05$ and $^{\dagger\dagger}p<0.01$ compared with epibatidine group.

1.5% bovine serum albumin and incubated overnight with the primary antibodies [rabbit anti-HO-1 (1:50; Chemicon International, Temecula, CA) and rat anti-FIRE (1:10; BD Pharmingen, San Diego, CA)]. Sections were incubated with fluorochrome-conjugated secondary antibodies (Rhodamine-anti-rabbit, Jackson immunoresearch, West Grove, PA; Alexa 647-anti-rat, Invitrogen, Carlsbad, CA) or peroxidase-conjugated secondary antibody (Chemicon International, Temecula, CA) followed by 0.06% diaminobenzidine and 0.2% H_2O_2 and hematoxylin counterstaining. Negative control sections were incubated without the primary antibodies. Sections were mounted and analyzed in a bright field light microscope (Zeiss Axioplan; Carl Zeiss GmbH, Jena, Germany) and in a confocal microscope (TCS SPE; Leica, Wetzlar, Germany).

Statistical analysis

Comparisons between experimental and control groups were performed by unpaired t test or Two-way ANOVA followed by Newman–Keuls test when appropriate. A value of $p<0.05$ was considered to be statistically significant.

3.Results

3.1.Effect of epibatidine treatment alone, or in the presence of the HO-1 inhibitor tin protoporphyrin (SnPP) in the formalin test

Licking time of mice injected with formalin in the hind paw during the first phase of the test was 90±5 s; this time was significantly reduced to 43±5 s when the animals were pre-treated with 4 µg/kg i.p. epibatidine 24 h before the test. The HO-1 antagonist SnPP administered via intraplantar (i.pl.) did not modify the licking time with respect to control. However, when the animals were injected in the paw with 400 nmol SnPP 5 min before the formalin test, the antinociceptive effect of epibatidine in the first phase of the test was partially but significantly reverted (**Fig 1A**).

When evaluating the second phase of the formalin test, epibatidine treatment also significantly reduced licking time in this phase, and SnPP completely blocked the antinociceptive effect of epibatidine (**Fig 1B**). Taken together these results indicate that peripheral HO-1 is implicated in the

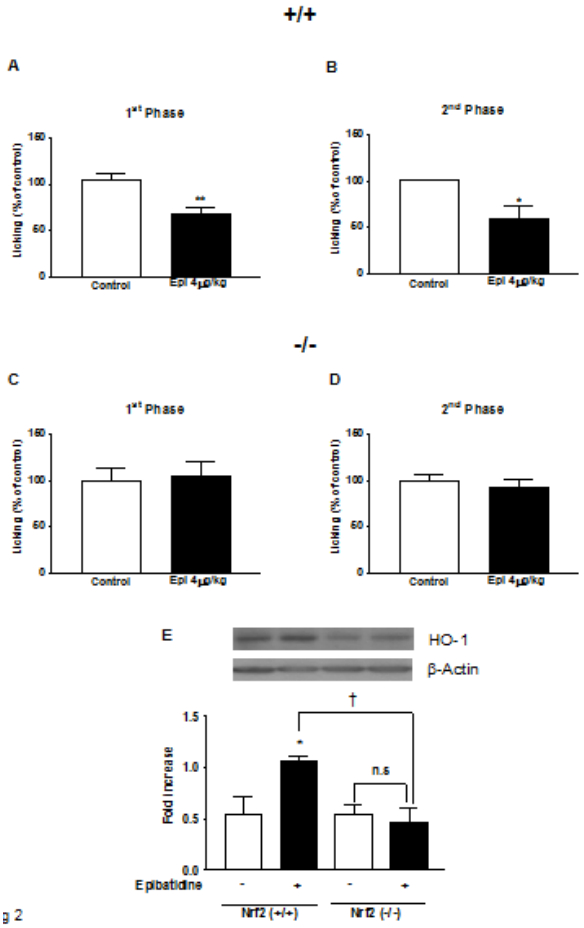

Figure 2. The antinociceptive effect of epibatidine was related to the transcription factor Nrf2 and HO-1 overexpression. Effect of treatment with vehicle or epibatidine (4 µg/kg, i.p.), 24 h before the formalin test, in wild-type (+/+) (A and B) and knockout mice for Nrf2 (-/-) (C and D) in the first and second phase of the formalin test. Values are normalized taking the mean of the control group as 100%; they are expressed as mean ± SEM (n = 6–8). Unpaired t test $^{*}p<0.05$ and $^{**}p<0.01$ compared with vehicle-treated control. (E) Western blot showing the expression of HO-1 in animals, treated with epibatidine (4 µg/kg, i.p.) or vehicle 24 h after the formalin test. β-actin is shown as a control below HO-1. Results represent the mean ± SEM of 3 independent experiments. Two-way ANOVA followed by Newman–Keuls test. $^{*}p<0.05$ compared with vehicle-treated control. $^{\dagger}p<0.05$ compared with wild-type treated with epibatidine.

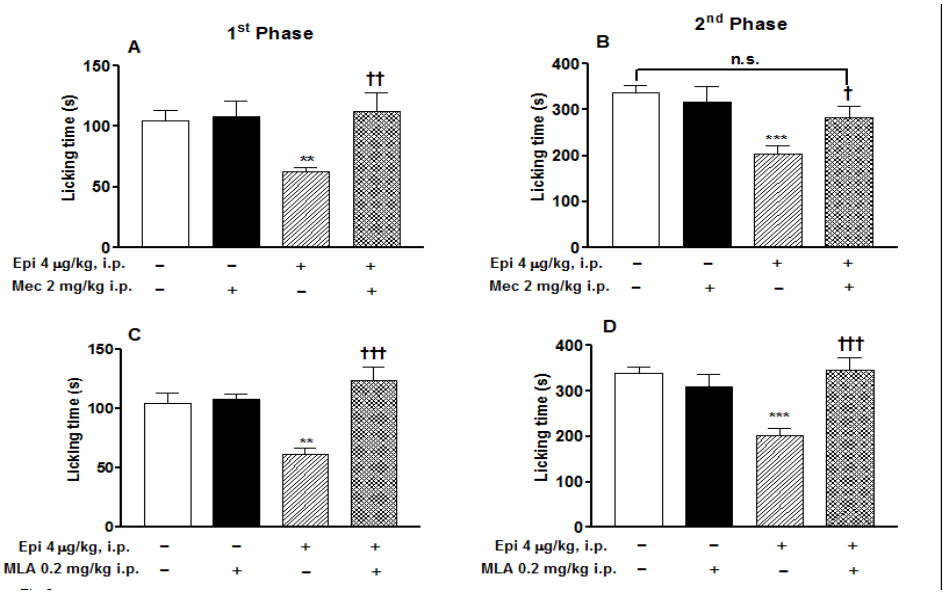

Figure 3. The antinociceptive effect of epibatidine was reverted by mecamylamine (a non-specific nAChR antagonist) and by methyllycaconitine (a specific α7 nAChR antagonist). Effect of pretreatment of *Swiss* mice with mecamylamine (2 mg/kg, i.p.) or methyllycaconitine (0.2 mg/kg, i.p.), on epibatidine (4 µg/kg, i.p.)-induced antinociception in the first (A and C, respectively) and second phase (B and D, respectively) of the formalin test. Each column represents the mean ± SEM (n = 8–10). Two-way ANOVA followed by Newman–Keuls test $^{**}p<0.01$ and $^{***}p<0.001$ compared with the vehicle-treated control. †$p<0.05$, † †$p<0.01$ and † † †$p<0.001$ compared with the epibatidine group.

antinociceptive effect of epibatidine during the first phase as well as during the second phase of the formalin test.

3.2.Effect of epibatidine in the formalin test in Nrf2 knockout mice

Since HO-1 induction can be mediated by the transcription factor Nrf2 and HO-1 induction has been described to have antinociceptive effects in the formalin test [27], we evaluated the effects of epibatidine treatment in the formalin test using control (+/+) and knockout (-/-) mice for Nrf2. Pre-treatment of +/+ mice with 4 µg/kg i.p. epibatidine resulted in significant reduction of licking time during the first and second phase of the formalin test (**Fig 2A and B**). When the same experiment was performed in Nrf2 knockout mice, epibatidine had no effect (**Fig 2C and D**). Therefore, HO-1 induction via Nrf2 could be responsible for the analgesic action of epibatidine.

3.3.Induction of HO-1 in epibatidine treated animals and its relation with the transcription factor Nrf2

To verify that epibatidine treatment was indeed overexpressing HO-1 via Nrf2, we took the plantar surface of the contralateral paws of the Nrf2 +/+ and -/- mice treated or untreated with epibatidine after the formalin test and quantified HO-1 by western-blot analysis. As represented in **Figure 2E**, treatment of the mice with a single dose of 4 µg/kg epibatidine i.p. raised HO-1 expression about 2-fold when measured 24 h after treatment. The same treatment in knockout mice for Nrf2 showed no increase in HO-1 expression.

3.4.Implication of nicotinic receptors in the antinociceptive effect of epibatidine in the formalin test

Next we wanted to evaluate if the antinociceptive effect of epibatidine described up to now was related to its action on nicotinic receptors. We first used the non-specific nicotinic receptor antagonist mecamylamine at 2 mg/kg i.p. given 30 min before epibatidine treatment; after 24 h, the animals were evaluated in the formalin test. **Figure 3** shows that mecamylamine treatment alone did not modify licking time of the mice during the first or second phase of the test (**Fig 3A and B**, respectively). However, when the animals were treated with mecamylamine and epibatidine, the antinociceptive effect of epibatidine was lost during both phases of the test. Then, we used the α7 selective antagonist MLA (0.2 mg/kg, i.p.) to establish whether α7 nicotinic receptors were implicated in the antinociceptive effect of epibatidine. **Figure 3** shows that MLA alone did not modify the nociceptive effect of the injection of formalin during the first or the second phase of the test. Furthermore, when the animals were treated with MLA plus epibatidine, the antinociceptive effect of epibatidine was completely lost in the two phases of the test (**Fig 3C and D**). These results proved that the effects observed for epibatidine in the formalin test were dependent upon α7 nicotinic receptors.

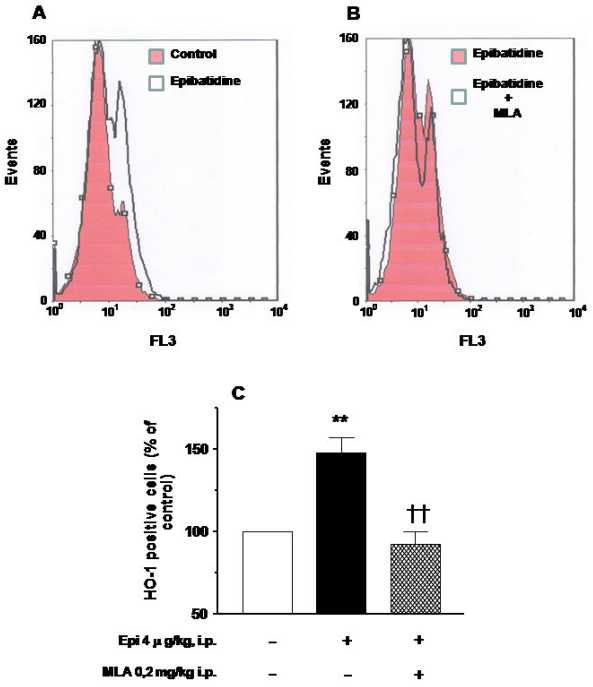

Figure 4. Treatment of animals with epibatidine augments HO-1 positive lymphocytes by activation of the α7 nAChR. (A and B) Two examples of the flow cytometry for HO-1 of lymphocytes of animals treated with saline (control), epibatidine and pretreated with methyllycaconitine 30 min prior to the treatment with epibatidine. (C) The histogram expresses the quantification of HO-1 positive cells of 3 independent experiments. Values are means ± SEM. Two-way ANOVA followed by Newman–Keuls test $^{**}p<0.01$ compared with the vehicle-treated control. $\dagger\dagger p<0.01$ compared with the epibatidine group.

3.5.Implication of white blood cells in the antinociceptive effect of epibatidine in the formalin test

Once we had proven that epibatidine induced HO-1 via α7 nicotinic receptors and the transcription factor Nrf2, we tried to determine which cells could be participating in this anti-inflammatory effect. Therefore, we treated the animals with vehicle, epibatidine, and MLA plus epibatidine. 24 h later, we dissected the spleen and extracted lymphocytes with the protocol shown in Methods. We analysed the presence of HO-1 in these cells by flow cytometry (**Fig 4**). Treatment with epibatidine increased the number of HO-1 positive lymphocytes (**Fig 4A**) and MLA reverted this effect to control levels (**Fig 4B**). The histogram in **figure 4C** shows the statistical results of these experiments. Moreover, when the animals were treated with MLA plus epibatidine, the increase of HO-1 positive cells was reverted.

We also analysed the presence of HO-1 in the spleen by immunohistochemistry and by immunofluorescence (**Fig 5**). HO-1 positive cells were found in the red pulp of the spleen (**Fig 5C**), not in the white pulp (**Fig 5D**). Treatment of animals with epibatidine augmented the number of HO-1 positive cells in this area (**Fig 5F**) with respect to control animals (**Fig 5E**), and MLA reverted this increase almost to control levels (**Fig 5G**). MLA alone did not have any effect (**Fig 5H**). The same results were obtained when HO-1 localization was determined by immunofluorescence (**Fig 5I-L**). However, when we analysed the immunofluorescence at higher magnifications, HO-1 was preferentially localized at the plasma membrane (red in **Fig 5M-P**) of macrophages since FIRE (F 4/80-like-receptor) (green in **Fig 5M-P**) showed a similar distribution to HO-1. These results point to an overexpression of HO-1 in the plasma membrane of white blood cells.

4.Discussion

In this study we revealed that peripheral activation of α7 nicotinic receptors and the consequent activation of the transcription factor Nrf2 leads to an overexpression of HO-1 in white blood cells, that can modulate inflammatory pain.

The antinociceptive effect of epibatidine was originally observed in the hotplate assay, where at 2.5 μg/kg caused significant analgesia [1]. Moreover, epibatidine has shown antinociceptive effects in various models of pain [3; 2; 4]. In all these cases, epibatidine was injected via i.p., i.t. or i.c.v. acutely before the test, and their effects were related to the central control of pain. In this context, our data point to a non acute, additional mechanism related to the overexpression of the anti-inflammatory protein HO-1. We recently showed that epibatidine can induce HO-1 after 24 h treatment *in vitro* [18]. Moreover, we also showed

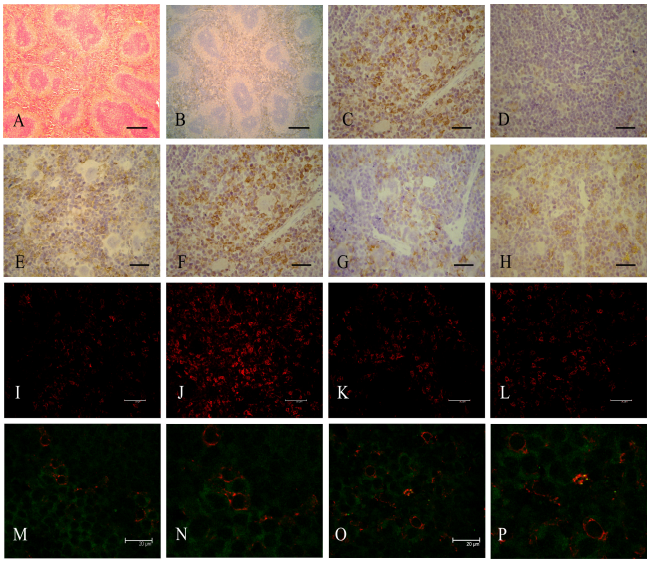

Figure 5. Haeme oxygenase-1 is localized in the red pulp of the spleen and is induced by activation of nAChRs. Photomicrographs of the mice's spleens: (A) hematoxylin-eosin stain, scale bar 400 μm; (B) HO-1 immunohistochemistry, scale bar 400 μm; (C) HO-1 immunohistochemistry of the red pulp, scale bar 50 μm; (D), HO-1 immunohistochemistry of the white pulp, scale bar 50 μm; (E-H) HO-1 immunohistochemistry of animals treated with vehicle, epibatidine, MLA plus epibatidine, and MLA, respectively, scale bar 50 μm; (I-L) HO-1 immunofluorescence of animals treated with vehicle, epibatidine, MLA plus epibatidine, and MLA, respectively, scale bar 50 μm; (M-P) HO-1 (red) and FIRE (green) double immunofluorescence, N and P are zooms of M and O, respectively, scale bar 20 μm.

that HO-1 overexpression has an anti-inflammatory and antinociceptive effect in the formalin test [27]. The antinociceptive effect of epibatidine was maintained 24 h after its injection; the fact that SnPP injection just before the formalin test reverted the antinociceptive effect of epibatidine, points to HO-1 as the responsible, at least in part, of its antinociceptive effect.

Nrf2 is a transcription factor related to the induction of HO-1 [26]. Our data show that epibatidine induces HO-1 through the transcription factor Nrf2, as we demonstrated by western-blot. In knockout animals for this transcription factor, epibatidine was unable to induce HO-1. Moreover, this loss of induction in Nrf2 knockout mice correlated with the loss of the antinociceptive effect shown by epibatidine in control mice. This finding is in agreement with others previously described that relate the activation of this transcription factor to anti-inflammatory effects in different models [30; 27].

nAChRs play important roles in modulating neurotransmitter release, cognition, sensory gating, pain transmission and anxiety. Thus, there has been increasing interest in the use of nicotinic agonists for pain therapy and treating neurodegenerative diseases [31]. The $\alpha4\beta2$ · and $\alpha7$ · nAChR subtypes are candidates for the central actions of nicotinic agonists because of their abundance in the CNS [10; 11]. Therefore, there is evidence that points to the $\alpha4\beta2$ nAChRs as the key receptor in antinociception induced by nicotinic agonists in the CNS [12; 2; 13; 14]. On the other hand, $\alpha7$ nAChRs, which are distributed extensively both in CNS and peripheral sites, are involved in many important functions [15-17], and are also related to antinociception. In this study, the antinociceptive effect of epibatidine seems to be related to its peripheral action on $\alpha7$ nAChRs because: i) MLA reverted the antinociceptive effect of epibatidine when injected 30 min before epibatidine, ii) SnPP completely blocked this antinociceptive effect and iii) treatment of animals with epibatidine produced an overexpression of HO-1 in white blood cells, that express nicotinic receptors including the $\alpha7$ subtype [32], and treatment with MLA before epibatidine injection blocked its overexpression.

Lymphocytes and macrophages-derived cytokines are able to directly or indirectly affect nociception and inflammation [33; 21]. TNF-α induces activity in nociceptive primary afferent fibers [34], and endo- or epineurial application of TNF-α to the sciatic nerve produces thermal hyperalgesia and mechanical allodynia [35; 36]. Blocking TNF-α [37], IL-1 receptors [38] or both [39] with neutralizing antibodies reduces nociception in mice. Furthermore, cytokine expression in T cells is enhanced in Nrf2 knockout mice [40]. Moreover, cytokine prodution such as TNF-α, IL-1β and IL-6 are increased after LPS treatment in knockout mice for $\alpha7$ nAChR compared to control mice [21]. Hence, a probable explanation for the effect seen in this study could be that the action of epibatidine on lymphocyte and macrophage · $\alpha7$ nAChRs activates the transcription factor Nrf2 which induces HO-1 and, HO-1 products or ROS arrestment by this enzyme could affect cytokine release. Furthermore, HO-1 seems to be anchored to the plasma membrane of macrophages as we have seen by immunofluorescence; the fact that HO-1 is present in the plasma membrane of these cells is in accordance with literature suggesting a local liberation of carbon monoxide that could inhibit the trafficking of Toll-like receptors to lipid rafts in the plasma membrane, inhibiting TLRs' signalling [41].

In conclusion, epibatidine showed a delayed antinociceptive effect in the formalin test 24 h after a single injection. This effect was mediated, at least in part, by peripheral $\alpha7$ nAChRs, located in white blood cells, that induced HO-1 expression via the transcription factor Nrf2. Therefore, the design of new drugs capable of selectively activating peripheral $\alpha7$ nAChRs could be an interesting strategy against nociception.

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V. DISCUSIÓN GENERAL

En este estudio se ha investigado los efectos neuroprotectores de distintos agonistas nicotínicos (nicotina y epibatidina) y de un modulador alostérico del receptor nicotínico e inhibidor de la acetilcolinesterasa (galantamina) en dos modelos de isquemia *in vitro*: la privación de oxígeno y glucosa (POG) en rodajas de hipocampo de rata y ratón y la exposición a los bloqueantes de la cadena respiratoria mitocondrial rotenona y oligomicina A en células cromafines bovinas. Además, se analizaron las rutas de señalización intracelular por las cuales estos compuestos ejercieron su efecto neuroprotector. Por otra parte, también se estudió el potencial efecto analgésico del agonista nicotínico epibatidina, inyectada 24 h antes de realizar el test de formalina, así como el mecanismo por el cual ejerce dicho efecto analgésico.

1. Neuroprotección

En un primer abordaje experimental, nos planteamos estudiar el potencial efecto neuroprotector de un inhibidor de la acetilcolinesterasa y potenciador alostérico del receptor nicotínico (Schrattenholz *et al.* 1996), la galantamina, y de un bloqueante selectivo de los receptores NMDA, la memantina. Estos dos fármacos se utilizan en la clínica para el tratamiento de pacientes con demencia tipo Alzheimer y vascular (Kurz *et al.* 2003, Reisberg *et al.* 2003).

El efecto protector observado por memantina (articulo 1), se relaciona con el mecanismo de acción ya conocido para este fármaco, el bloqueo no competitivo de los receptores NMDA. De esta forma, se impediría la excitotoxicidad inducida por glutamato que se produce, en el modelo de POG-reoxigenación, durante la hora de privación de oxígeno y glucosa. De este modo, bloqueando la excitotoxicidad por glutamato, obtenemos una protección de un 40% a la concentración de 10 µM. Como control positivo, utilizamos el MK-801, ya que también es un bloqueante no competitivo de estos receptores de glutamato. Así, a la concentración de 1 µM, obtuvimos una protección del 42%, corroborando que los bloqueantes de los receptores NMDA son protectores en el modelo de POG-reoxigenación en rodajas de hipocampo de rata. Estos datos concuerdan con otros datos de la literatura, en los que se observa protección con MK-801 y memantina tanto en modelos de isquemia “in vivo” (Seif el Nasr *et al.* 1990), como en modelos de hipoxia “in vitro” (Seif el Nasr et al. 1990, Gorgulu *et al.* 2000).

Ante una situación de 60 min de POG y 180 min de reoxigenación, la galantamina ofreció protección significativa a las concentraciones de 5 y 15 µM en rodajas de hipocampo (artículo 1). El estudio de su mecanismo neuroprotector fue más

complejo, pues, además de los mecanismos ya conocidos para este fármaco (potenciador alostérico del receptor nicotínico e inhibidor moderado de la acetilcolinesterasa), se han descrito otros. Por ejemplo, la galantamina puede actuar como captador de radicales libres (Traykova *et al.* 2003); además, a concentraciones altas, también es capaz de bloquear los receptores nicotínicos (Maelicke *et al.* 2000).

En un segundo estudio (artículo 2), cambiamos el protocolo de POG-reoxigenación, reduciendo el tiempo de POG a 15 minutos y el de reoxigenación de 60 minutos. En estas condiciones experimentales prácticamente no hay liberación de LDH pero puede cuantificarse una lesión importante mediante el doble marcaje con las sondas fluorescentes ioduro de propidio, IP, y Hoechst. Con el uso de la microscopía de fluorescencia, además, podemos analizar por separado la lesión en las distintas áreas del hipocampo (artículo 2). De esta forma, corroboramos que a la concentración de 15 µM la galantamina protege, tanto en el área CA1 como en CA3 del hipocampo. Además, esta protección está mediada por receptores nicotínicos ya que se revirtió cuando las rodajas se coincubaron con mecamilamina, un antagonista no específico de los nAChR.

Además, el efecto neuroprotector de la galantamina fue revertido por un antagonista de la vía PI3K/Akt. La quinasa Akt es capaz de regular por fosforilación la actividad de, por una parte GSK-3β, y por otra. la de la proteína pro-apoptótica Bad. Además, es capaz de reducir la producción de radicales libres y de disminuir la inducción de iNOS producida por la POG. La activación de los receptores NMDA por el glutamato liberado después de un episodio isquémico, se ha relacionado con el aumento en la expresión de iNOS en rodajas de cerebro a través de la activación Ca^{2+}-dependiente de NFκB (Cardenas *et al.* 2000). Nosotros hemos visto que la galantamina es capaz de reducir en un 46% la liberación de glutamato tras la POG en rodajas de hipocampo de rata. De esta forma, la galantamina puede proteger por un mecanismo doble: reduciendo la excitotoxicidad de glutamato y, de manera secundaria, reduciendo la expresión de iNOS debida a esa excitotoxicidad.

Por otra parte, la POG produce la defosforilación de Akt y, por tanto, disminuye su actividad (Zhou *et al.* 2008). Akt es un quinasa que, entre otras proteínas, controla la actividad de GSK-3β y Bad fosforilandolas e inhibiendolas. Cuando Akt se defosforila por la POG, también disminuye la fosforilación de GSK-3β y Bad, activándose ambas. Hay muchos datos en la literatura que demuestran que la inhibición de GSK-3β es protectora frente a diversos estímulos tóxicos (Manji *et al.* 1999, Bhat *et al.* 2004,

Kelly *et al.* 2004, Endo *et al.* 2006). La fosforilación e inhibición de Bad también se ha demostrado que es neuroprotectora (Zhou et al. 2008). Así, el pretratamiento con galantamina, restauró a niveles basales la fosforilación de Akt y por consiguiente, el de GSK-3β y el de Bad, inhibiéndolas y, por lo tanto, ofreciendo neuroprotección. Dado que mecamilamina inhibió las acciones de galantamina a nivel de fosforilación de Akt, GSK-3β y Bad, lo más probable es que la galantamina esté activando esta vía de supervivencia celular a través de su interacción con los receptores nicotínicos. Estos datos concuerdan con los de la literatura, en los que relacionan la ruta de la PI3K/Akt con la activación de receptores nicotínicos y la protección (Kihara *et al.* 2001, Shimohama & Kihara 2001, Kihara et al. 2004, Arias et al. 2005).

Los nAChR están muy concentrados en el hipocampo, en el tálamo y en la corteza cerebral (Clarke *et al.* 1985, Wada *et al.* 1989, Hogg *et al.* 2003). Últimamente, ha cobrado mucho interés el hecho de que varios subtipos de nAChR pudieran estar involucrados en los mecanismos neuroprotectores frente a distintos estímulos tóxicos que producen daño neuronal. En estudios realizados en cultivos neuronales, los agonistas nicotínicos ofrecen efectos neuroprotectores frente a glutamato (Gahring *et al.* 2003, Sun et al. 2004), deprivación de factores tróficos (Yamashita & Nakamura 1996), β-amiloide (Gahring et al. 2003, Kihara et al. 1997) e hipoxia (Hejmadi et al. 2003). Actualmente, hay muchas dudas sobre qué subtipo de nAChR está involucrado en los mecanismos neuroprotectores. A pesar de esto, el subtipo α7 está emergiendo como una clara diana para el desarrollo de fármacos neuroprotectores. En este sentido, utilizamos el modelo de POG-reoxigenación para ver si la protección que muestran los agonistas de los nAChR está asociada a algún subtipo concreto de estos receptores (artículo 3). Así, la nicotina, a las concentraciones probadas (10, 30 y 100 μM) protegió frente a la POG-reoxigenación, y esta protección fue mediada por activación de los nAChR, ya que la mecamilamina revirtió este efecto neuroprotector. Como disponíamos de ratones transgénicos para el subtipo α7 de los nAChR, medimos la protección ofrecida por nicotina en ratones +/+ y -/- para este subtipo de receptor nicotínico (artículo 3). Observamos que la nicotina ofrecía neuroprotección frente a la POG-reoxigenación en rodajas de hipocampo de ratones controles (+/+), sin embargo, esta protección desaparece en los ratones con ablación para el subtipo α7 (-/-). Estos resultados supusieron una clara evidencia de

que los receptores nicotínicos α7 desempeñan un papel importante en la neuroprotección ofrecida por nicotina en rodajas de hipocampo sometidas a POG-reoxigenación. Además, refuerzan el papel de los receptores α7 en los efectos neuroprotectores de los agonistas nicotínicos frente a la toxicidad inducida por glutamato (Donnelly-Roberts et al. 1996, Kaneko et al. 1997) e hipoxia (Hejmadi et al. 2003) en cultivos primarios neuronales, frente a β-amiloide en células de neuroblastoma humano SHSY-5Y (Arias et al. 2004, Arias et al. 2005) y frente a tapsigargina en células cromafines bovinas (Arias et al. 2004).

Con el objetivo de explorar los mecanismos a largo plazo por los que los receptores nicotínicos ofrecen neuroprotección, utilizamos cultivos primarios de células cromafines bovinas, ya que nos permiten tiempos de preincubación más largos que los de rodajas de hipocampo. En este sentido, utilizamos como modelo de isquemia "in vitro", un modelo desarrollado en el laboratorio. Dicho modelo consiste en combinar rotenona, un inhibidor del complejo I de la cadena respiratoria mitocondrial, y oligomicina-A, un inhibidor del complejo V (ATPasa) (artículo 4). Decidimos combinar estos dos tóxicos por el hecho de que por sí solos, y en 24 horas de exposición, no producían un aumento significativo de la muerte celular medida a través de la liberación de LDH al medio extracelular. Así, empleando este estímulo citotóxico pudimos observar que, la epibatidina, era capaz de proteger de manera concentración-dependiente. Además, en el efecto neuroprotector de la epibatidina participaba la salida de calcio del retículo y la posterior activación de PKC, que a su vez iniciaba la ruta neuroprotectora de las MAPK. La mayor novedad de este artículo, fue que la activación de receptores nicotínicos puede inducir la sobre-expresión de HO-1, una enzima antioxidante y relacionada con efectos neuroprotectores frente a diversos estímulos tóxicos (Poss & Tonegawa 1997, Kim et al. 2005, Nimura *et al.* 1996, Parfenova *et al.* 2006).

Teniendo en cuenta estos 4 artículos, podríamos concluir que, tras la activación de los nAChR, se activan rutas neuroprotectoras que, a corto plazo, son capaces de frenar la muerte celular por fosforilación directa de distintas quinasas relacionadas con la muerte celular y la apoptosis (GSK-3β, Bad). Además, a largo plazo, son capaces de inducir la síntesis de proteínas tales como HO-1, que por su capacidad antioxidante y neuroprotectora, puede reducir el daño mitocondrial producido por los ROS y, así, prevenir la apoptosis celular.

2. Dolor

Las especies reactivas de oxígeno pueden modular la nocicepción. De hecho, la administración de antioxidantes como la vitamina C, E, o analgésicos, disminuye la producción de ROS inducida por estímulos nociceptivos (Rokyta *et al.* 2003, Rokyta *et al.* 2004). La administración de N-acetilcisteína (NAC) en el núcleo del trigémino, mitiga completamente el dolor producido por la administración en la región orofacial de formalina (Viggiano *et al.* 2005).

Con este pensamiento surgió la idea de que, no solo fármacos antioxidantes, sino una enzima con un potente efecto antioxidante como la HO-1 pudiera tener un efecto antinociceptivo en el modelo de dolor de la inyección de formalina en la pata (artículo 5). Los resultados de este trabajo muestran que la inducción de HO-1, a través del factor de transcripción Nrf-2, puede modular la nocicepción. La HO-1 sólo fue efectiva durante la segunda fase del modelo de formalina, y no en la primera fase. Esto indica que esta enzima antioxidante puede modular principalmente la nocicepción de origen inflamatorio. Estos resultados están de acuerdo con datos de la literatura que demuestran un efecto anti-inflamatorio de la HO-1 (Mohri *et al.* 2006, Poss & Tonegawa 1997, Sato *et al.* 2006). Además, el hecho de que la HO-1 se encuentre aumentada en enfermedades inflamatorias (Mohri et al. 2006, Sato et al. 2006) podría significar que esta enzima participa como un agente anti-inflamatorio endógeno. Además, en este trabajo demostramos que la inducción de HO-1 se produce en células circulantes (macrófagos, monocitos, linfocitos), que curiosamente, son de las primeras células que acuden cuando se produce inflamación periférica.

Esta hipótesis la continuamos explorando, utilizando la epibatidina, ya que sabíamos que era capaz de inducir la HO-1 y, esto podría añadir un mecanismo nuevo al ya conocido efecto antinociceptivo a nivel central de epibatidina (artículo 6). El hallazgo principal de este artículo es que la epibatidina es capaz de inducir la HO-1 en células blancas del sistema inmune (macrófagos y linfocitos), a través de la activación del receptor nicotínico α7. Este subtipo de receptor, está adquiriendo mucha importancia, no solo porque es capaz de mediar efectos neuroprotectores, sino porque además es capaz de mediar efectos anti-inflamatorios. Así, Wang y colaboradores en su artículo publicado en 2003 definen a este subtipo de receptor como esencial en la regulación de la inflamación (Wang et al. 2003). En este artículo demuestran que el tratamiento de macrófagos con nicotina disminuye la liberación de TNF-α inducida por LPS. Además, este efecto lo revierten utilizando oligonucleótidos antisentido contra

el receptor α7. Estos autores también demuestran que los ratones transgénicos para este receptor tienen niveles de TNF-α en suero más altos que los ratones controles. Nuestros resultados avalan la hipótesis de que el receptor α7 tiene un papel muy importante en la inflamación.

Además, en este artículo demostramos que la HO-1 se encuentra anclada a la membrana plasmática de los macrófagos. Nuestros resultados concuerdan con la hipótesis que proponen Nakahira y colaboradores, en la que el monóxido de carbono liberado de forma local por la reacción catalizada por la HO-1, puede inhibir el tráfico de receptores Toll-like (TLR) hacia la membrana, inhibiendo así la señalización pro-inflamatoria que se produce a través de ellos (Nakahira *et al.* 2006).

Así pudimos poner de manifiesto que, la epibatidina, además del ya descrito efecto analgésico a corto plazo mediado por receptores nicotínicos a nivel central, también posee un efecto analgésico a más largo plazo mediado por su acción sobre receptores $\alpha 7$ periféricos. Este último se relaciona con un efecto anti-inflamatorio mediado por la inducción de HO-1 en células blancas del sistema inmune.

VI. CONCLUSIONES

CONCLUSIONES DE ESTA TESIS DOCTORAL

De los resultados que presentamos en esta memoria, se pueden extraer las siguientes conclusiones:

1. La galantamina, a las concentraciones de 5 y 15 µM, y la memantina, a la concentración de 10 µM, protegen las rodajas de hipocampo de rata sometidas a POG-reoxigenación.

2. La galantamina protege frente a la POG-reoxigenación por un mecanismo dual, por una parte inhibe la inducción de iNOS y reduce la producción de ROS, y por otra activa la ruta de supervivencia de PI3K/Akt que, a su vez, inhibe a GSK-3β y Bad, fosforilandolos.

3. La nicotina protege las rodajas de hipocampo de rata y ratón sometidas a POG-reoxigenación a través de la activación del receptor nicotínico α7.

4. La epibatidina protege a las células cromafines bovinas frente a la lesión producida por estrés oxidativo, por un mecanismo en el cual participa la salida de calcio del retículo y posterior activación de PKC que, a su vez, activa la ruta de señalización de las MAPK, produciendo la inducción de HO-1.

5. La inducción de HO-1, a través del factor Nrf-2, tiene un efecto antinociceptivo en la segunda fase del modelo de formalina; dicho efecto se relaciona con la actividad anti-inflamatoria de esta enzima a nivel periférico.

6. La epibatidina induce la sobre-expresión de HO-1 a través de la activación de los receptores α7 de macrófagos y linfocitos; dicha sobre-expresión se correlaciona con el efecto antinociceptivo observado en el modelo de formalina.

VII. BIBLIOGRAFÍA

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